Vol. 53, 1975

ıcta Haematologica

International Journal of Haematology
Journal International d Hématologie
Internationale Zeitschrift für Hämatologie

34

Official Organ of the European Division of the International Society of Haematology

S. Karger · Basel München · Paris · London · New Y

S. Karger · Basel · München · Paris · London · New York · Sydney
Arnold Böcklin-Strasse 25, CH–4011 Basel (Switzerland)

Printed in Switzerland by National-Zeitung AG, Basel

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Acta haemat. 53: 1–16 (1975)

Haarzell-Leukämie

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Abstract. Chronical course, splenomegaly and pancytopenia are characteristic for hairy cell leukemia. The main feature are so-called hairy cells in peripheral blood. This cells were investigated by cytological, cytochemical, histological and electron microscopical methods. Special cytoplasmic inclusions seem to be specific for this cell type.

Key Words
Cytochemistry
Electron microscopy
Hairy cell leukemia
Leukemic reticuloendotheliosis
Reticuloses

Die sogenannte Haarzell-Leukämie (HZL) ist bisher im deutschen Schrifttum nur selten beschrieben worden [23]. Die meisten Publikationen stammen aus den USA und führen für dieses Krankheitsbild unterschiedliche Bezeichnungen (leucemic reticuloendotheliosis, neoplastic lymphoid reticulum cells in the blood, chronic reticulo-lymphocytic leukemia, hairy cell leukemia, reticulum cell leukemia). Die Besonderheit dieser Leukämie ist das Auftreten sogenannter Haarzellen oder 'hairy cells' im peripheren Blut [3, 5, 6, 10, 20, 28, 30, 32, 34, 38].

Die HZL wird erst seit der Beschreibung von Bouroncle *et al.* [3] als einheitliches Krankheitsbild aufgefasst. In den meisten Untersuchungen wurde sie bisher unter der Bezeichnung «leucemic reticulo-endotheliosis» beschrieben. Vor dem Jahr 1958 wurde sie jedoch nicht exakt gegen das leukämisch verlaufende Retikulosarkom abgegrenzt (Bezeichnungen im amerikanischen Schrifttum: leucemic reticulum cell sarcoma [27, 39], histiocytic lymphoma [31], histiocytic leukemia [26]). Erstmalig verglich Plenderleith [30] 12 Fälle von HZL mit 100 Retikulosarkomen, bei denen er jedoch niemals Haarzellen

[1] Mit Unterstützung durch Mittel der Schutzkommission beim Bundesministerium des Innern.

[2] Frau A. H. Lenz und Frau O. Caesar danken wir für die hervorragende technische Hilfe.

im peripheren Blut fand. Er wies ausserdem auf den relativ kurzen Krankheitsverlauf der Retikulosarkeme hin, der im Mittel 9 Monate beträgt. Schnitzer und Kass [33] bestätigen dies. Auch elektronenmikroskopische Befunde unterstreichen den Unterschied zwischen dem leukämisch verlaufenden Retikulosarkom und der HZL [11, 33]. Es ist anzunehmen, dass die von Gosselin et al. [13] anhand von 49 Fällen beschriebene Retikuloendotheliose nur mit Vorbehalten als HZL angesehen werden darf. Die Kasuistik weist hier auf 10 Fälle mit ausgedehnten spezifischen Hautinfiltraten hin, ein Befund, der bei HZL äusserst selten ist.

Das namengebende Strukturmerkmal der Haarzellen besteht in der Ausprägung langer, «haarartiger» Zytoplasmafortsätze. Neben diesen typischen Haarzellen gehören Panzytopenie, Infiltration von Knochenmark, Milz, Leber und Lymphknoten, ausgeprägte Splenomegalie und ein chronischer Verlauf zum Bild der HZL. Sie ist eine Erkrankung des mittleren und höheren Lebensalters (60% sind älter als 50 Jahre) und wurde bisher bei Kindern nicht beobachtet. Männer erkranken wesentlich häufiger als Frauen (etwa 5:1). Insgesamt sind etwa 2% aller Leukämien HZL.

Seit 1958 sind zirka 170 Fälle von HZL beschrieben worden [1, 3, 5, 6, 8, 10, 19, 20, 28–30, 32, 34, 35, 38]. Gegenstand der vorliegenden Arbeit sind zytochemische und elektronenmikroskopische Untersuchungen an Haarzellen, sowie histologische Befunde an Knochenmark, Milz, Leber und Lymphknoten bei 4 Fällen von HZL, die wir in den Jahren 1972 und 1973 beobachteten.

Kasuistik

Fall 1. 54jähriger Mann, 1967 Beinvenenthrombose, 1969 Thromboserezidiv, anschliessend Marcoumarbehandlung. August 1970 Aufnahme in auswärtigem Krankenhaus wegen Anämie und Leukopenie. Ulzerationen im Bereich der Mundschleimhaut, Hämaturie. BSG 135/152. Antikoagulantientherapie abgesetzt. Therapie: Bluttransfusionen. In der Folgezeit weiterhin Abgeschlagenheit, Müdigkeit, Kopfschmerzen, Schwindel und Gewichtsabnahme von 9 kg in 2 Monaten. Wiederaufnahme wegen Panzytopenie, ausgeprägter Splenomegalie und starker Hämaturie. Sternalpunktion: Punctio sicca. Beckenkammbiopsie (Medizinische Universitätsklinik Freiburg): Infiltration von atypischen, teils lymphoiden, teils retikulären Zellen mit Verdrängung des normalen Markes und Faserbildung. Verlegung in die Medizinische Universitätsklinik Freiburg.

Aufnahmebefund (Februar 1972): Diffuse petechiale Blutungen, kleine Retinablutung. Leber nicht vergrössert, Milz 5 cm unter dem Rippenbogen. Keine Lymphknotenvergrösserung. Laborwerte: Tabelle I. Zytochemischer Befund: Tabelle II. Therapieversuch mit Vincristin und Decortin. Weiterhin Bluttransfusionen. Im Verlauf nekrotisierende Angina und schwerste, nicht zu beherrschende Bronchopneumonie. Exitus letalis. Obduktion verweigert.

Fall 2. 59jähriger Mann, 1969 Grippe, in der Folgezeit Klagen über Leistungsminde

Tabelle I Laboratoriumsbefunde bei Fällen 1–4

	Fall 1	Fall 2	Fall 3	Fall 4
BSG, mm	136/150	94/110	95/129	40/100
Hb, g%	7,2	11,0	10,1	9,2
Erythrozyten, Mio	2,5	4,4	3,47	2,46
Leukozyten	2 600	6 500	5 600	2 800
HZ, %	84	80	70	84
Thrombozyten	80 000	40 000	51 000	87 000
Sternalpunktat	Punctio sicca	90% HZ	70% HZ	über 90% HZ
Paraproteine	0	0	0	0

Tabelle II Vergleichende zytochemische Befunde an Lymphozyten, Haarzellen und Monozyten

	Lymphozyt	Haarzelle	Monozyt
Peroxidase und Chloracetatesterase	0	0	0 – +
PAS	+	+	+
Unspezifische Esterasen	+	+	+ + +
Saure Phosphatase	+	+ – + +	+ +
β-Glucuronidase	+	0	+

rung. Müdigkeit und Dyspnoe. Gelegentlich Hämoptoe. Aufnahme November 1971 in der Medizinischen Universitätsklinik Freiburg.

Befund: *Leber* handbreit unter dem Rippenbogen, Milz 15 cm unter dem Rippenbogen. Keine Lymphknotenvergrößerung. Laborwerte: Tabelle I. Zytochemische Befunde: Tabelle II. Lymphangiografie o. B. Laparoskopie: Infiltration von Leber und Milz. Histologisch: Tumorzellinfiltrate bei lymphoretikulärer Systemerkrankung. Beckenkammbiopsie: Infiltration von atypischen retikulären Zellen, meist in lockerer Anordnung, deutliche Faservermehrung. Weitgehende Verdrängung des blutbildenden Marks. Therapie: Vincristin und Decortin, später Velbe, Natulan und Endoxan (bis August 1972), dann Endoxan bis Juli 1973. Zweite Aufnahme September 1972 wegen Bronchopneumonie. Schnelle Rückbildung unter Antibiotika. Entlassung nach einem Monat. Juli 1973 erneute Aufnahme wegen heftigster linksseitiger Oberbauchschmerzen. Klinisch: Milzinfarkt. Versuch der Milzbestrahlung mit Telekobalt. Zunehmende Kachexie, hämorrhagische Diathese und Bronchopneumonie. Exitus letalis.

Obduktionsbefund: Hepatomegalie (2450 g), Splenomegalie (2290 g) mit 7 cm grossem, zerfallendem, anämischem Infarkt. Femur- und Wirbelmark: *Normales Fettmark und rotes blutbildendes Mark weitgehend durch landkartenartige, weisslich-graue Herde ersetzt.* Blutungen in Milz, Knochenmark und Lunge. Schleimhautblutung in Magen und Harnblase. Lymphknoten nicht wesentlich vergrössert. Lymphknoten des Lun-

genhilus Infiltrate atypischer Zellen Milz Ausgedehnte Tumorzellinfiltration teilweise durch Blutungen überlagert Leber Dichte rundkernige Infiltrationen der Portalfelder, nur spärliche Infiltration der Sinusoide Cholestase Todesursache Schwere areaktive Pneumonie und Zeichen einer massiven Rechtsherzbelastung

Fall 3 39jähriger Mann, früher häufig Anginen und rheumatische Beschwerden 1971 und 1972 Gewichtsverlust von 10 kg Leistungsabfall Herbst 1972 mehrfach Hauthämatome nach Bagatelltraumen Januar 1973 starke Nachblutung nach Zahnextraktion Wegen Thrombopenie Aufnahme in die Medizinische Universitätsklinik Freiburg Februar 1973

Befund Deutliche Blässe Keine Lymphknotenvergrosserung Leber 2 Querfinger unter dem Rippenbogen, Milz 25 cm unter dem Rippenbogen Lymphografie Vergrösserung und teilweise Zerstorung mit Speicherdefekten der Lymphknoten paraaortal Laborwerte Tabelle I Zytochemische Befunde Tabelle II Beckenkammbiopsie Infiltration von atypischen Zellen und weitgehende Verdrängung des blutbildenden Markes Deutliche Faservermehrung Häufig Plasmazellen und Gewebsmastzellen Therapie Vincristin und Decortin Hierunter Grössenabnahme der Milz und Rückgang der Lymphknotenvergrösserung bei Lymphangiografiekontrolle Entlassung Erneute Aufnahme wegen Panzytopenie im Mai 1973 Befund Deutlicher Ikterus Übriger Befund unverändert Zytostatische Therapie mit Vincristin Mustargen und Natulan Rückgang der Milzvergrosserung um 6 cm Rückbildung des Ikterus Unverändert Panzytopenie In der Folgezeit hämorrhagische Diathese und Pneumonie Septische Temperaturen Exitus letalis

Obduktionsbefund Ausgeprägte Vergrösserung der Leber (2630 g) und der Milz (3980 g) Zahlreiche anämische Infarkte mit ausgedehnten Hämorrhagien der Milz Verbackene Lymphknotenpakete Mark von Sternum Wirbelkörper und Femur durch homogene weisslich gelbe Herde verdrängt Im Femurmark landkartenartige Nekrosen Blutungen in Milz Lymphknoten Peritoneum Nierenbecken, Endokard Haut und Magenschleimhaut In beiden Lungenoberlappen areaktive hämorrhagische Pneumonie Todesursache Akutes Rechtsherzversagen bei starker areaktiver Pneumonie Dichte Infiltrationen von atypischen Zellen in Milz Lymphknoten und Knochenmark, deutliche Faservermehrung des Knochenmarkes In der Leber diffuse Infiltration sowohl der Portalfelder als auch der Sinusoide, hier auch in doppelreihigen Strängen angeordnet

Fall 4 59jähriger Mann, nie ernstlich krank Leistungsminderung im letzten halben Jahr Anfang April 1973 Panzytopenie Wegen Verdachtes einer malignen Systemerkrankung Aufnahme in der Medizinischen Universitätsklinik Freiburg Juli 1973

Befund Blässe *Milz 4 cm unter dem Rippenbogen, keine Lymphknotenvergrösserung* Leber nicht vergrössert Laborwerte Tabelle I Zytochemische Befunde Tabelle II Beckenkammbiopsie Weitgehende Verdrängung des normalen blutbildenden Marks durch atypische Zellen deutliche Faservermehrung Therapie Vincristin Decortin Nach 3 Wochen Entlassung in unverändertem Zustand Erneute Aufnahme im August 1973 wegen Lungeninfarktes Deutliche Belastungsdyspnoe EKG Zeichen eines akuten Cor pulmonale Röntgenthorax Mediastinalverbreiterung Verdacht auf Lymphome Therapieversuch mit Adriamycin Antibiotische Therapie Keine Besserung. Kein Nachweis von Pilzen im Sputum Unter Herzkreislaufversagen Exitus letalis

Obduktionsbefund 10 × 5 × 3 cm grosse hämorrhagische Nekrose zwischen Aortenbogen und Arteria pulmonalis links im vorderen Mediastinum In diesem Bereich grosser Parietalthrombus der Aorta descendens Grosse bröckelige Gefässausgüsse in den Oberlappenästen der linken Pulmonalarterie Gesamter linker Lungenoberlappen hämorrha

gisch infarziert 5 cm langer Embolus im Oberlappenast der rechten Pulmonalarterie 3 cm grosser Lungeninfarkt im rechten Oberlappen Ausgedehnter Pilzbefall mit Candida albicans im Bereich des Mediastinums der Lippen des Kehlkopfes und des Antrum ventriculi Dichte Durchsetzung von Pilzfäden im Bereich der Parietalthrombose der Aorta descendens, in den Blutgerinnseln der *Pulmonalarterien sowie in den hämorrhagischen Infarkten der Lunge* Weiterhin ausgedehnte weisslich-gelbe Infiltrate in Wirbelsäule, Sternum und Beckenmark

Mikroskopische Befunde

Lichtmikroskopie

Bei der HZL findet man im Blut und Knochenmark einen besonderen Zelltyp, der durch eine auffallend unregelmässige Begrenzung gekennzeichnet ist Nach *panoptischer Färbung* lassen sich «haarförmige» Zytoplasmaausläufer erkennen Die Zellen sind in der Regel grösser als Lymphozyten und besitzen ovale bis leicht eingebuchtete Kerne mit lockerem Chromatin Das blasse, graublaue Zytoplasma erscheint wabig und enthält manchmal vakuolenartige Strukturen Azurgranula sind nicht nachweisbar Dagegen kommen stäbchenförmige Gebilde vor, die einen kristallartigen Eindruck machen (Abb 1, 5)

Zytochemische Färbungen

Die Peroxydase Reaktion [36] fiel obligat negativ aus Die PAS Färbung [14] brachte neben einer diffusen Tingierung immer feine Granulationen (Abb 1c) In einem Fall waren auch gröbere Granula vorhanden, nie jedoch PAS positive Schollen oder grobtropfige Einlagerungen Die unspezifische Esterase-Reaktion [22] zeigte die übliche Basisaktivität, die β Glukuronidase [24] einen negativen Ausfall Die saure Phosphatase Reaktion [12] ist stets in wechselnder Stärke vorhanden (Abb 1c) Neben einer diffusen Anfärbung zeigte sie auch ein deutlich granuläres Muster Die *Knochenmarkshistologie* (Methode nach BURKHARDT [4]) ergab einen gesteigerten Zellgehalt der Markräume mit weitgehender Verdrängung des Fettmarks Eine normale Erythropoese fand sich nur noch spärlich in Form kleiner Erythroblasteninseln (Abb 2) Die Granulopoese fehlte nahezu vollständig Nur vereinzelt liessen sich in Balkchennähe Promyelozyten mit Hilfe der für sie typischen Chloracetatesterase Reaktion [14] auffinden In den Markräumen lagen Haarzellen meist in lockerer Anordnung und zeigten teils lymphoiden teils retikulären Charakter Auch hier waren in ihrem Zytoplasma stäbchenförmige Gebilde nachweisbar (Abb 3) Zwischen ihnen kamen vereinzelt Plasmazellen und Mastzellen vor Mit Hilfe der Versilberungsmethode [4]

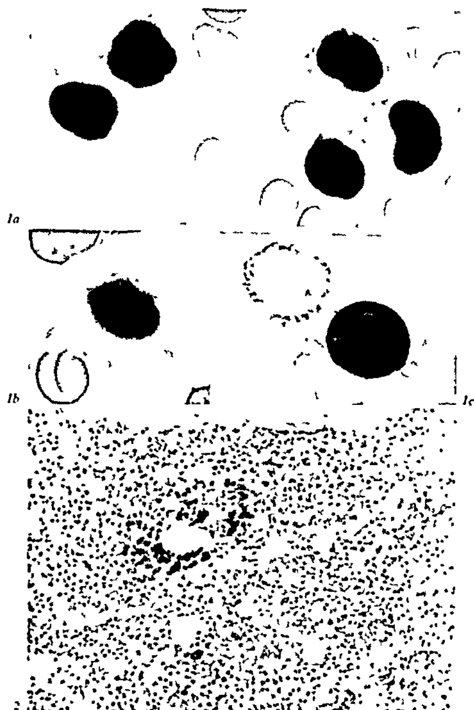


Abb. 1 Haarzellen im peripheren Blut. *a* Pappenheim-Färbung. *b* Saure Phosphatase-Reaktion. *c* PAS-Färbung. ×1200

konnte eine deutliche Faservermehrung dargestellt werden, die abhängig von der Dauer des Krankheitsverlaufes zunahm. Dabei schienen Erythroblasteninseln ausgespart zu sein (Abb. 5). Die Punktionszylinder der *Leber* zeigten bei regelmässigem Läppchenaufbau eine dichte Infiltration der Periportalfelder mit atypischen Zellen, die bohnenförmige bis ovale, chromatinreiche Kerne mit kleinen Nukleolen aufwiesen. Dazwischen lagen vereinzelt Lymphozyten und Plasmazellen. Die Gallengänge waren intakt, die Sinusoide mässig ausgeweitet. Hier wurden atypische Zellen beobachtet, die häufig in dichten, zweireihigen Kolonnen angeordnet waren (Abb. 4). Einzelnekrosen fanden sich im Parenchym selten. In der *Milz* waren vereinzelt noch Malpighische Körperchen nachweisbar. Die rote Pulpa war mässig gestaut und zeigte teils dichte, teils lockere Infiltrate atypischer Zellen. Die nur unwesentlich vergrösserten *Lymphknoten* wiesen keine Lymphfollikel mehr auf. Neben Retikulumzellen, Lymphozyten und Plasmazellen kamen atypische Zellen vor, die an verschiedenen Stellen die Lymphknotenkapsel durchbrachen und das angrenzende Fettgewebe infiltrierten.

Elektronenmikroskopie

Im elektronenmikroskopischen Bild erscheinen die Haarzellen unregelmässig gestaltet und besitzen stets Pseudopodien unterschiedlicher Länge (Abb. 6). Der relativ grosse Kern zeigt oft eine deutliche Impression und ist von einer gleichmässigen perinukleären Zisterne umgeben, die vereinzelt Kernporen aufweist. Das Zytoplasma ist auffallend reich an Mikrofilamenten und Polyribosomen, die sich zu den sogenannten Ribosomen-Lamellen-Komplexen [19] zusammenlagern können (Abb. 7, 8). Nur selten treten Profile des granulären ER in Erscheinung. Stets sind Zentriole vorhanden, die durch zahlreiche Satelliten und mächtige Kondensationszentren gekennzeichnet sind (Abb. 9). Mitochondrien mit deutlichen Cristae liegen gruppenweise im Zytoplasma. Häufig kommen »multivesicular bodies« vor, ebenso grosse Vakuolen mit heteromorphem Inhalt (Abb. 9, 10). Das Plasmalemm der Zellen zeigt vereinzelt Mikropinozytosevesikulationen. Oft ist an der extrazellulären Seite filamentäres Material in kleinen Klumpen angelagert. Die Pseudopodien der Haarzellen berühren sich untereinander und nehmen auch Kontakt mit den Membranen der Erythrozyten auf.

Abb. 2. Knochenmark mit unterschiedlich dichter Infiltration von Haarzellen. Reste der normalen Blutbildung in der Mitte. Giemsa-Färbung, ×250

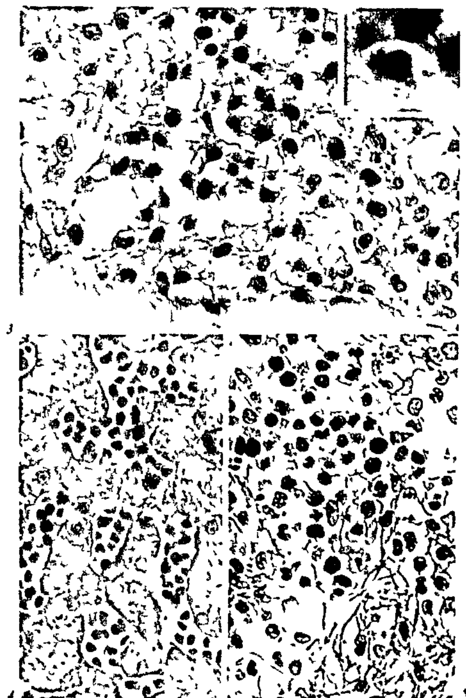


Abb. 3 Knochenmark stärker vergrößert. Die Haarzellen sind netzartig angeordnet. Stäbchenförmiger Einschluss im Zytoplasma einer Haarzelle oben rechts. Giemsa-Färbung × 800

Diskussion

Der Beginn einer Erkrankung an HZL ist meistens nicht eindeutig zu bestimmen, da die klinischen Symptome uncharakteristisch sind. Die meisten Patienten klagen über Leistungsminderung, Müdigkeit, Neigung zu Infekten, sowie Druckgefühl und Schmerzen im linken Oberbauch. Letztere sind durch die Splenomegalie erklärbar, die in 75–100% der Fälle gefunden wird [3, 6, 10, 18, 20]. Auch unsere Patienten hatten eine ausgeprägte Milzvergrösserung. Im Verlauf der Erkrankung treten häufig Milzinfarkte auf, die bei zwei unserer Patienten erhebliche Beschwerden verursachten. Eine Lebervergrösserung wird nur in der Hälfte der Fälle [3, 6, 10], eine Vergrösserung der Lymphknoten nur selten beobachtet. Einmal konnte bei einer Lymphangiographie ein paraaortaler Lymphknotenbefall mit weitgehender Strukturzerstörung festgestellt werden, obwohl oberflächliche Lymphknoten nicht zu tasten waren. Dieser Befund deckt sich mit den pathologischen Veränderungen, die durch Lymphangiographie von BOIRON *et al* [2] in 3 von 4 Fällen, von FLANDRIN *et al* [10] in 6 von 9 Fällen und von CATOVSKY *et al* [6] in 2 von 6 Fällen beobachtet wurden. In 2 Fällen beschrieb PLENDERLEITH [30] eine meningeale Aussaat.

Die Panzytopenie – bisher in allen Fällen der HZL beobachtet – schwankt in ihrem Ausmass und hat als wichtiges Symptom zu gelten. Erythropoese, Granulopoese und Thrombopoese können unterschiedlich betroffen sein, oft ist nur ein Zellstrang betroffen. Bei unseren Patienten lagen die Hb-Werte zwischen 7,2 und 11,0 g%, die Erythrozytenzahlen zwischen 2,5 und 4,4 Mio und die Thrombozytenzahlen zwischen 40 000 und 80 000. FLANDRIN *et al* [10] beobachteten bei 66% der Patienten Thrombozytenzahlen unter 100 000. In 3 von 4 Fällen fanden sie eine vermehrte Sequestration in der Milz als Ausdruck eines Hyperspleniesyndroms. Wir stellten in 2 Fällen mit zunehmender Milzvergrösserung ein Absinken der Thrombozytenzahlen fest. Dabei muss jedoch beachtet werden, dass im Verlauf der HZL eine zunehmende Infiltration des Knochenmarkes erfolgt, die die Thrombopoese zurückdrängt. FISCHER [9] zeigte, dass bei ausgeprägter Splenomegalie keineswegs ein Hyperspleniesyndrom vorliegen muss. Die Leukozytenwerte lagen in unseren Fällen zwischen 2600 und 6500. Der Anteil der

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Abb. 4 Reihenartige Anordnung der Haarzellen in den Lebersinusoiden. Giemsa-Färbung × 400

Abb. 5 Faserbildung im Knochenmark, die im Bereich einer Erythroblasteninsel fehlt. Versilberung nach Gomori × 400

10 µm

6

1 µm



Abb 8 Haarzelle (Ausschn tt) »R bosomen-Lamellen Komplex« im Querschn tt × 56 000

Haarzellen machte hierbei zeitweise 70 84% aus Diese ausgeprägte Granu lozytopenie dürfte für die Infektanfälligkeit der Patienten verantwortlich sein In der Literatur werden nur in Einzelfällen Leukozytosen von mehr als 15000 angeführt [10 20 30 38] Nur in einem Fall ist eine erhebliche Leukozytose von 134000 beschrieben worden [3]

D e Sternalpunktion ist vielfach unergiebig BOURONCLE *et al* [3] be schrieben ein Punctio s cca in 77% der Fälle Der Grund dürfte in der ver mehrten Faserbildung des Knochenmarkes zu suchen sein Da bei der HZL d e Anzahl der Haarzellen im peripheren Blut schwankt kann bei geringem Auftreten das Krankheitsbild zunächst verkannt werden Die charakteri stische Struktur der Zellen ist jedoch in der panoptischen Färbung unver kennbar und zeigt s ch besonders gut im Phasenkontrast [1 34 35] In 2 Fällen konnten wir stäbchenförmige Gebilde in den Zellen nachweisen die auch FLANDRIN *et al* [10] und KATAYAMA *et al* [19] kürzlich beschrieben haben Offensichtlich handelt es sich hierbei um d e gleichen Strukturen die im elektronenm kroskopischen B ld als sogenannte Ribosomen Lamellen Komplexe in Erscheinung treten [18] Wir beobachteten diese Gebilde sowohl im peripheren Blut wie auch im Knochenmark wo sie bis jetzt noch nicht beschrieben worden sind

Abb 6 Elek ronenm kroskopische Übersichtsaufnahme der Haarzellen × 5000

Abb 7 Haarzelle mit sogenannten »R bosomen-Lamellen-Komplexen« Unten rechts Haarzelle im L htm kroskop × 9900

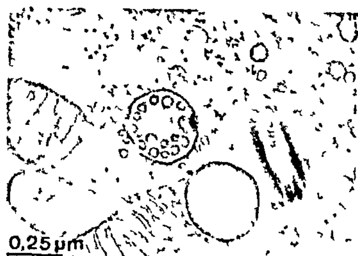


Abb. 9. Haarzelle (Ausschnitt). Zentriol und «multivesicular body». × 38 000

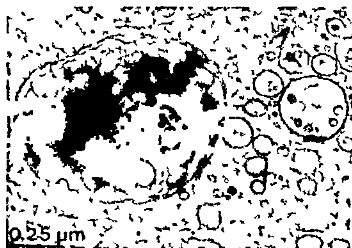


Abb. 10. Haarzelle (Ausschnitt). Vakuole mit heteromorphem Inhalt und «multivesicular body». × 56 000

Zytochemisch ergaben sich bei der PAS-Reaktion neben einem diffusen auch ein granulärer, bei der unspezifischen Esterase ein feingranulärer Ausfall. Eine wichtige Rolle spielt die saure Phosphatase-Reaktion, die in den Haarzellen stets positiv ist. YAM *et al.* [37] fanden ein für Haarzellen typisches Fehlen der Tartrathemmung der sauren Phosphatase. Dieser Befund

wurde von FLANDRIN *et al* [10] KATAYAMA *et al* [17] und CATOVSKY *et al* [6] bestätigt YAM *et al* [38] konnten zeigen, dass es sich bei dieser sauren Phosphatase um das Isoenzym (Nr 5) handelt, das bei normalen Lymphozyten und bei chronischen lymphatischen Leukämien allenfalls in ganz geringem Masse vorkommt [21] KATAYAMA *et al* [17] beschrieben dies auch für einen Teil der Retikulumzellen in Hodgkin-Infiltraten

Im Knochenmark geht mit dem deutlich gesteigerten Zellgehalt eine Verdrängung des Fettmarkes einher Reste der normalen Granulo-, Thrombo- und Erythropoese können noch vorhanden sein, stets ist jedoch ein vermehrter Fasergehalt anzutreffen

Unsere histologischen Befunde aus Leber, Milz und Lymphknoten stimmen mit den Beschreibungen der Literatur überein [6, 10 18] Die elektronenmikroskopischen Befunde an Haarzellen bestätigen die phasenkontrastmikroskopischen Beobachtungen von TRUBOWITZ *et al* [35] dass dieser Zelltyp eine ausgeprägte Eigenmotilität besitzt, die auch im peripheren Blut nicht aufgegeben wird Lange, bereits im Lichtmikroskop sichtbare Pseudopodien und zahlreiche Mikrofilamente sind morphologische Strukturmerkmale dieser amöboiden Bewegungsfähigkeit Die sogenannten Ribosomen-Lamellen Komplexe [6 10, 16 18] dürften jedoch in anderem Zusammenhang zu deuten sein Ihr lichtmikroskopisches Äquivalent bilden wahrscheinlich die stäbchenartigen Zytoplasmaeinschlüsse der Haarzellen Die Aufklärung ihrer Struktur und Genese ist Gegenstand weiterer Untersuchungen Phagozytose konnte von uns elektronenmikroskopisch nicht nachgewiesen werden Die lichtmikroskopisch sichtbare, granuläre Reaktion der sauren Phosphatase weist jedoch in diese Richtung So können die Vakuolen heteromorphen Inhalts als Phagolysosomen oder autophagische Vakuolen angesehen werden Auffallend ist das spärliche Auftreten des granulären ER Bei den im Zytoplasma frei liegenden elektronendichten Granula von Ribosomenkaliber durfte es sich zum Teil um β Glykogen handeln, eine Annahme, die durch die positive PAS Reaktion gestützt wird

Die licht und elektronenmikroskopischen Befunde der HZL lassen heute eine sichere Abgrenzung zu der chronischen lymphatischen Leukämie, den Lymphosarkomen sowie zu den leukämisch verlaufenden Retikulosarkomen zu In der Mehrzahl der Fälle wird ein chronischer Verlauf beschrieben Im Einzelfall muss jedoch immer mit Komplikationen durch die Panzytopenie (Hämorrhagien Infektionen) gerechnet werden Die Angaben uber therapeutische Massnahmen sind in der Literatur nicht einheitlich Nach CATOVSKY *et al* [6] und FLANDRIN *et al* [10] scheinen Zytostatika wenig erfolgversprechend zu sein Auch wir konnten kein Ansprechen auf eine zytostatische

Therapie beobachten Guten Erfolg sehen mehrere Autoren in der Splenektomie [10, 20], die CATOVSKY *et al* [6] sogar als das Mittel der Wahl bezeichneten Sicher wird diese Therapie in Fällen mit einem Hypersplenie-syndrom das Krankheitsbild günstig beeinflussen

Zusammenfassung

Chronischer Verlauf Splenomegalie und Panzytopenie lassen die Haarzell Leukämie oft schwer von anderen lymphoretikulären Systemerkrankungen abgrenzen Die Besonderheit dieser Leukämie ist das Auftreten von sogenannten Haarzellen im peripheren Blut An diesem Zelltyp wurden zytologische, zytochemische, histologische und elektronenmikroskopische Untersuchungen durchgeführt Hierbei konnten sowohl lichtmikroskopisch als auch elektronenmikroskopisch Strukturmerkmale nachgewiesen werden, die für diesen Zelltyp spezifisch sind

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Sonderdruckbestellungen an Dr W MÖBIUS, Medizinische Universitätsklinik D-78 *Freiburg i Br* (BRD)

Acta haemat 53 17-24 (1975)

Phagocytic Activity of Leukaemic Blasts

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Abstract Phagocytic activity of leukaemic blasts in 20 adults and 16 children suffering from acute leukaemia was studied *in vitro* by the use of ferrioxidsaccharate Most frequently phagocytosing blasts were encountered in myelomonocytic leukaemias The morphological character of positive blasts resembled often that of monocytoid cells In lymphoblastic and reticular leukaemias phagocytic ability of blasts was an exceptional finding

Key Words
Acute leukaemia
Ferrioxidsaccharate phagocytosis
Phagocytosis in leukaemia

Numerous papers appeared on phagocytosis in acute leukaemia, paying attention mainly to mature granulocytes [2, 4, 6, 7, 16, 17, 19–21, 23 25] Phagocytic capability of leukaemic blasts was studied less intensively and on small groups of patients only and no uniform results were reported so far [2, 5, 8 9, 11–14, 17, 18, 22, 24] As regard the blasts in healthy bone marrow it is believed that they are not capable of phagocytosis [9 10 12 13]

In our paper phagocytic activity of leukaemic blasts has been followed up in order to explain two questions are leukaemic blastic cells capable of phagocytosis and if this is the case, to what degree? Would the presence of phagocytosis contribute to the classification of acute leukaemia?

Method and Material

For the study of phagocytosis ferrioxidsaccharate (Ferrocid Aesca, Austria) was used, a substance easily ingested by normal micro- and macrophages [15] Under sterile conditions ferrioxidsaccharate was added in excess to the heparinized

blood so that the final concentration was 5 mg of iron on 100 ml of blood. The blood sample was incubated 1 h in 37 °C. Supernatant plasma with buffy coat was centrifuged and from the sediment blood smears were made. They were stained according to Perls method and counterstained with neutral red. At least 400 blasts were evaluated in 4 slides and the percentage of blasts with blue granules of iron was assessed. In order to estimate the intensity of phagocytosis cells were divided into 3 classes according to the content of iron ingested. Parallel evaluation of cellular morphology was performed on identical panoptically stained smears.

In addition neutrophils containing ingested iron were recorded. In blood samples of control persons under the same laboratory conditions 34–94 cells/100 phagocytes contain ingested ferrioxidsaccharate [15a].

Cytochemical examination of leukaemias included conventional methods for neutral lipids, peroxidase, non-specific esterases (normal and blocked by natrium fluorid), further PAS reaction and staining for nucleoli.

Samples for electron microscopy from 13 patients were fixed by glutaraldehyde and after osmification embedded into araldite. Sections were not contrasted and were examined in the microscope JEM 7.

Phagocytosis was studied in 36 patients: 20 adults and 16 children. Acute haemoblastosis was classified as myeloblastic in 12 cases, as myelomonocytic in 8 cases, lymphoblastic and reticular in 12 cases. The precise classification was not possible in 4 cases (table I).

Results

In 20 cases out of 36, blasts did not show any phagocytosis or only very few cells contained several small grains of iron (table II). In 7 cases with weak phagocytosis less then 10% of blasts were positive and the intensity of phagocytosis was minor or medium (see also table I). In 9 cases phagocytosis was marked, more frequently encountered in adults. Adult leukaemias were markedly positive in 35% of cases contrary to 12% in children group. Individual cases are presented in table I.

Phagocytosing blasts did not possess any special morphology (fig. 1, 2). They were larger than inactive blasts with scarce plasma or presented monocytoid nucleus of more mature character than negative cells. Similarly, in electrone microscope blasts with round nucleus and small rim of plasma did not contain ferrisaccharate, while cells with monocytoid nucleus did. Grains of stainable iron in optical microscope were frequently of unequal size, often localized at the periphery of the cell in weakly phagocytosing blasts and also above the nucleus in more intensive phagocytosis. Some of larger grains of stainable iron were lighter and transparent. All this resembled more the monocytic type of phagocytosis of ferrioxidsaccharate than the neutrophilic type [15]. Corresponding

Table I Cytochemical classification of acute leukaemia and percentage of phagocytosing blasts and neutrophils in individual cases

Type of leukaemia	Case No	Sex	Age years	Percentage of positive blasts and intensity of phagocytosis	Percentage of positive neutrophils
Myeloblastic	1	m	13	0	9
	2	f	76	0	48
	3	f	36	0	63
	4	f	35	0	72
	5	f	58	0	89
	6	m	13	0	21
	7	m	57	1	20
	8	m	29	1	62
	9	m	53	2+	25
	10	f	43	2+	59
	11	f	73	3+	0
	12	f	46	24++	20
Myelomonocytic	1	m	13	5+	46
	2	m	9	8+	64
	3	m	59	15+++	100
	4[1]	m	2	24+++	52
	5	m	56	50++	97
	6	m	77	52+++	100
	7	f	17	57++	94
	8	f	40	72+++	60
Lymphoblastic, reticular	1	f	8	0	0
	2	m	7	0	0
	3	m	52	0	2
	4	f	60	0	4
	5	f	18	0	57
	6	m	7	0	37
	7	f	2	0	38
	8	m	5	0	50
	9	m	6	0	75
	10	f	36	0	98
	11	f	11	0	35
	12	f	8	3++	100
Non-differentiated	1	m	8	0	95
	2	m	14	2++	24
	3	m	53	22+	44
	4	f	13	49+++	10

[1] Leukaemic cells possessed the character of relatively mature monocytic cells. Clinical course and autopsy findings were consistent with the diagnosis of acute leukaemia.



Fig 1 Non differentiated leukaemia in a 13 year old girl 49% positive blasts
Fig 2 Myelomonocytic leukaemia in a 77 year old man 52% positive blasts

Table II Phagocytic activity of leukaemic blasts

Phagocytosis	Positive blasts, %	Adults	Children	Total
Negative	2	10	10	20
Weak	2 8	3	4	7
Marked	15 72	7	2	9

findings in electron microscope were phagocytic vacuoles of various sizes, some of them thickly, some thinly filled with ferrioxidsaccharate (fig 3)

In samples with a high percentage of phagocytosing cells the estimation of positive blasts was no problem for the observer only in rare cases intensive clumping of phagocytosing more sticky cells prevented to assess even higher percentage of positive blasts since the remaining isolated blasts in the smear often were negative (case No 3 with myelomonocytic leukaemia in table I) In cases with very low percentage of positive cells differentiation between monocytoid blasts and positive



Fig. 3. Electron microscope photograph from the same patient as in figure 2. Ferrous saccharate ingested in cytoplasma of a monocytoid blast. Adherent particles on cellular membrane and formation of phagocytic vacuoles. × 760.

atypical monocytes or myelocytes was not always easy and could be a cause of erroneous result. In these cases thorough study of panoptically stained smears was especially important.

A correlation of the presence or absence of phagocytosing blasts with the cytochemical type of leukaemia is demonstrated in table I. Positive blasts were found in all cases of myelomonocytic leukaemia. In the majority of these leukaemias phagocytosis was marked. Contrary to that phagocytosis was an exception in lymphoblastic and reticular leukaemias.

In two thirds of leukaemic patients mature neutrophils demonstrated normal or busy phagocytosis, especially in myelomonocytic leukaemias [table I]. In two lymphoblastic leukaemias no cells at all were capable of phagocytosis. No relation between phagocytosis and the number of leukocytes or the absolute number of blasts was found.

In the majority of adult and in some children leukaemias phagocytosis was examined repeatedly and identical results were obtained with one exception In the blood sample of patient No 9 (table I) with myeloblastic leukaemia during the relaps after 3 months 12% positive blasts were found

Discussion

For the study of phagocytosis in acute leukaemia various material was used as indian ink [14, 24], microbes [5, 9, 11], yeast [2, 7, 17], latex particles [8] or white cells attacked by antinuclear factors [13]. In our laboratory ferrioxidsaccharate was chosen for two reasons mainly: it is easily ingested by the cell and the whole procedure does not require *any elaborate equipment and can be performed in any haematological* laboratory.

From our results it may be assumed that capability of phagocytosis, at least of inert particles as ferrisaccharate, is not an exception in blasts of acute leukaemia The question remains, why some blasts have this ability and others have not It might be a matter of mere chance, as BESSIS [1] is convinced, that under certain conditions every cell is capable of phagocytosis Or it might be primarily a matter of serum factors [7] This seems to be less probable, since in majority of our cases with negative blasts, neutrophils did phagocytose However, this alternative cannot be excluded on the basis of our results and needs further elucidation Finally, phagocytosis might be the property of the leukaemic cell, given by its different cellular origin The validity of the last alternative is supported by our results, showing relationship with the cytochemical type of leukaemia Phagocytosing blasts were present especially in myelomonocytic leukaemias Also the morphological character of positive blasts resembled often that of monocytes The small number of cases examined do not permit us to claim that phagocytosis occurs in all cases of myelomonocytic leukaemia in general However, similar observations may be found in the literature [3, 18, 24] Phagocytic activity of monocytic blasts must be connected with some special function and metabolism of these leukaemic cells, as for example with their high content of lysozyme [3] The exceptional finding of phagocytosis in lymphoblastic and reticular group is not surprising, as phagocytosis is not a property of the lymphoid cell More or less diverse morphology of phagocytosing and non phagocytosing blasts in some cases of leukaemias is

suggestive for the presence of a mixed population of blasts, consisting either of cells of various maturity or cells of different cellular origin

The small number of patients investigated does not warrant us to suggest the examination of phagocytosis as a functional test completing cytochemical classification of leukaemias An accessory question arises whether phagocytosis might be a way of transport of some pharmacologically important compounds into the leukaemic cell

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Acta haemat 53 25-29 (1975)

Quantitative Studies of Macrophages in Blood Cultures in Hodgkin's Disease

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Abstract In Hodgkin's disease blood cultures showed a close relationship between the rate of blastic transformation of PHA stimulated lymphocytes and the number of macrophages appearing in unstimulated cultures Despite individual variations in single cases the yield of macrophages agreed closely with the lymphocyte transformation rate Results are discussed in connection with hypothesis on the origin of macrophages in culture

Key Words
Blood culture
Hodgkin's disease
Lymphocyte transformation
Macrophages
Phytohaemagglutinin

In chronic lymphocytic leukaemia (CLL) previous experiments [14] disclosed a direct relationship between the percentage of blastic transformation of lymphocytes stimulated *in vitro* with phytohaemagglutinin (PHA) and the amount of macrophages which appeared in cultures without PHA Such relationship has been regarded as not casual, since, however the low number of both blast cells and macrophages in cases of CLL with high white blood cell count could be referred to an excessive dilution of normal lymphocytes and monocytes by leukaemic cells [10] we have now investigated the same problem in Hodgkin's disease, a condition in which a frequent if not constant decrease of PHA blastic transformation of lymphocytes [1 6 9 11 16 19] is associated with a normal or low white blood cell count

Materials and Methods

Leucocytes obtained from venous blood of 17 normal subjects and 40 patients with Hodgkin's disease in various stages, either treated or untreated were cultured *in vitro* The cultures were performed as described elsewhere [20] in TC 199 Well

come with antibiotics and 15% autologous serum after mechanical defibrination of the blood and separation of leucocytes with gelatin (Plasmagel Roger Bellon). The unpurified buffy coat was added to the medium so as to obtain a final cellular concentration of 2×10^6/ml, the whole culture volume being 15 ml. In all cases blood cultures were performed both with and without PHA Wellcome (0.02 ml/ml of medium). An 18×18 mm glass slide was put at the bottom of the culture tubes according to BENNETT and COHN [2].

The percentage of blastic transformation of lymphocytes was assessed morphologically after 3 days, whereas the macrophages adherent to the slides were counted after 6 days of culture. Macrophage counts were carried out on eight different areas, of 1 mm² each, and the mean arithmetical value was calculated.

Results

The results are summarized in table I. In healthy subjects the percentage of blast cells in PHA stimulated cultures was 80.06 ± 6.09%, the

Table I. Blast cells and macrophages in blood cultures from normal subjects and patients with Hodgkin's disease

Diagnosis	Number of cases	Blastic transformation of lymphocytes with PHA %	Number of macrophages/mm² without PHA
Normal subjects	17	80.06 (65–90) SD 6.09	96.17 (42–210) SD 50.5[illegible]
Hodgkin's disease			
All cases	40	46.55 (4–89) SD 23.82	37.02 (0–119) SD 35.40
Cases with PHA blastic transformation under 30%	12	17.58 (4–30) SD 8.57	1.83 (0–6) SD 2.82
Cases ranging from 30 to 60%	11	41.36 (31–57) SD 7.9[illegible]	23.54 (1–70) SD 20.90
Cases over 60%	17	70.35 (61–89) SD 6.62	70.59 (21–119) SD 32.63

number of macrophages in cultures without PHA was 96.17±50.58/mm³. In Hodgkin's disease the blastic transformation of lymphocytes in PHA stimulated cultures was lower than in normal subjects, i.e. 46.55±23.82%, and the number of macrophages appearing in unstimulated cultures was also relatively low (37.02±38.40 mm³). However, individual results varied within a very large range, as shown by the high standard deviations. Therefore we divided our patients in three groups according to the degree of PHA blastic transformation of lymphocytes (table I). In cases with a low blastic transformation (under 30%, mean value 17.58+8.57%) also the number of macrophages was very low (1.83±2.82/mm³). In cases with a blastic transformation ranging from 30 to 60% (mean value 41.36±7.96%) the number of macrophages was somewhat higher than in the previous group (23.54±20.90 mm³). In cases with an high, almost normal percentage of blast cells (over 60%, mean value 70.35+6.62%) the number of macrophages in the cultures without PHA (70.59+32.63/mm³) approached that observed in normal subjects.

Discussion

In *in vitro* blood cultures from patients with Wiskott-Aldrich syndrome and Hodgkin's disease BLAESE *et al.* [5] observed that the yield of macrophages obtained from patients was generally lower than in controls. In skin window preparations in Hodgkin's disease REBUCK *et al.* [15] reported a reduction of the overall cellularity, whilst, using a similar technique, GHOSH *et al.* [7] found a copious cellular migration (over 90% of the cells being macrophages) either in normal subjects and in Hodgkin's disease.

Our results show that a direct relationship exists between the percentage of PHA induced blastic transformation of lymphocytes and the number of macrophages appearing in unstimulated cultures in Hodgkin's disease. The cases with a low percentage of blast cells showed also a low number of macrophages, whilst a high transformation of lymphocytes was constantly associated with a high yield of macrophages, as we already observed in CLL [14].

The evaluation of the present findings cannot be univocal, as the interpretation of the mechanism of decreased PHA lymphocyte transformation in Hodgkin's disease has led to different hypotheses. (1) If it is assumed that a plasmatic factor is involved [11, 17], it could be suggested that the

same factor also inhibits the development of macrophages in blood cultures (2) Others [3, 13] believe that a depletion of the PHA responsive circulating T-lymphocytes is responsible for the low blastic transformation in some cases of Hodgkin's disease If it is also assumed that lymphocytes could transform into macrophages in blood cultures [4, 8], the lymphopenia often observed in advanced cases of Hodgkin's disease (which are those with a low PHA lymphocyte transformation) [9, 16, 19] could also account for the decreased yield of macrophages (3) If the source of macrophages in culture were only the monocytes [12, 18] the number of these should be reduced in peripheral blood of patients with Hodgkin's disease with a low blastic transformation of lymphocytes This observation, however, has not been reported Therefore, it seems that hypothesis 1 and 2 are more likely to be correct

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Acta haemat. 53: 30-36 (1975)

Insoluble PHA – A B-Cell Mitogen in Man?[1]

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Abstract. Blood lymphocytes of normal individuals and of patients with chronic lymphocytic leukemia were evaluated for their blastogenic response to insoluble PHA. In at least preferential stimulation of normal B lymphocytes was observed, whereas leukemic lymphocytes were almost insensitive. This result supports the hypothesis of a functionally defective B lymphocyte population in chronic lymphocytic leukemia.

Key Words: B lymphocytes · Lymphocytes stimulation · Lymphocytic leukemia · PHA

Various cell surface markers have been used to characterize lymphocyte populations [1, 3, 15, 18, 19, 21], which differ in their response to some mitogens [4, 6]. In mice a variety of experimental evidence suggests that phytohemagglutinin (PHA) acts only on T-lymphocytes [12]. B lymphocytes are also sensitive to PHA but only if the mitogen is presented in an insoluble form [7]. We have examined the response of normal and leukemic human peripheral blood lymphocytes to insoluble (sepharose-bound) PHA in comparison to the soluble mitogen.

Materials and Methods

Cyanogen bromide activated sepharose (Sepharose 4B, Pharmacia, Uppsala) was used. The coupling procedure with PHA P (Difco Lab., Detroit) was carried out as described by GREAVES *et al.* [7]. The loaded beads were then kept at 4 °C overnight and used for the experiments on the next day. For determining the amount of bound PHA the mitogen was labelled with iodine 125 (Farbwerke Hoechst, Frankfurt; specific activity 8-15 Ci ^{125}I mg I) by the chloramine T method [11].

[1] Supported by the Fond zur Förderung der wissenschaftlichen Forschung No. 1769.

Lymphocytes from 12 patients with chronic lymphocytic leukemia (CLL) were investigated. The total white blood cell count ranged from 12×10^3 to 324×10^3/μl with 69–99% lymphocytes. Three patients were under treatment with corticosteroids and 9 were treated with chlorambucil and corticosteroids. As controls we used lymphocytes from 10 hospitalized persons who showed a normal blood picture and did not exhibit immunological abnormalities. The lymphocytes were cultured under standard conditions [9].

Lymphocyte suspensions without sepharose beads as well as cultures incubated with PHA free beads served as controls. In some experiments the culture supernatants were obtained and tested for mitogenic activity. These supernatants had no stimulatory effect. Replicate cultures were performed in 4 patients, the results differed up to 25%.

^{3}H-thymidine incorporation into DNA was evaluated after 12 h labelling and the stimulation index calculated as follows:

$$\frac{\text{counts/min in the presence of mitogen}}{\text{counts/min in the absence of mitogen}}$$

The method of WYBRAN *et al.* [22] with some modifications was employed for obtaining lymphocyte suspensions enriched with B- or T-lymphocytes. After rosetting, the lymphocyte suspensions were loaded on a Ficoll-Hypaque gradient (1 ml cell suspension to 1 ml flotation medium). The tubes were centrifuged at 400 g for 20 min and the pellet as well as the interface fraction were collected using a Pasteur pipette. After determining the percentage of rosette forming lymphocytes (at least 3 bound red cells to one lymphocyte) the SRBC were hemolyzed by a short hypotonic shock, the cells washed twice in Hanks solution, resuspended in TC 199 and cultured with insoluble or soluble PHA under culture conditions.

Results

In 3 experiments with ^{125}I-PHA it was shown that about 90% of the PHA was bound to the sepharose beads when using 10 mg PHA. Using larger amounts of PHA the percentage of bound PHA decreased (fig. 1). The optimal dose for soluble PHA determined in previous experiments was 20 μg PHA/ml culture for normal as well as leukemic lymphocytes. The dose-response curve of insoluble PHA was similar in controls and patients: the optimal concentration in both groups was 40 μg bound PHA/ml culture (fig. 2).

In the presence of soluble or insoluble PHA, the maximal PHA-response of normal lymphocytes occurred at 72 h. Under both conditions leukemic lymphocytes showed a delayed response, with low values at day 3 and higher values at days 5 and 7 (fig. 3). Therefore the experiments were performed with an incubation period of 5 days.

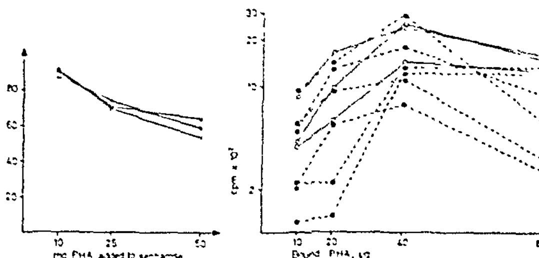


Fig. 1. Dosis dependence of bound PHA to sepharose beads (3 experiments): PHA was labelled with [131]I and various amounts incubated with sepharose beads.

Fig. 2. Stimulation of normal (o–o) and leukemic (●–●) lymphocytes by various amounts of sepharose-PHA

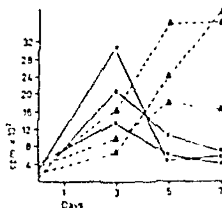


Fig. 3. Stimulation of normal (×–×) and leukemic (▲–▲) lymphocytes by sepharose-PHA after various incubation times.

Lymphocytes from 10 controls and 12 patients with CLL were tested. The blastogenic response, as measured by ^{3}H-thymidine incorporation, is shown in table I. In the controls, soluble PHA was more effective than the insoluble mitogen. The average stimulation index was approximately three times higher. In patients with CLL there were 2 groups. Three patients with a very low transformation in the presence of soluble PHA showed a better transformation by insoluble PHA. The

Table I Transformation of normal and leukemic lymphocytes by insoluble PHA in comparison to soluble PHA

	Soluble PHA		Insoluble PHA	
	counts/min	stimulation index	counts/min	stimulation index
Controls				
1	94,515	138	21,079	30
2	85,800	170	11,449	22
3	73,150	133	11,101	39
4	49,634	83	21,516	36
5	46,709	618	1,365	18
6	43,540	70	32,909	53
7	34,321	70	22,814	46
8	28,816	58	8,882	18
9	24,726	34	3,734	5
10	18,361	14	5,649	5
CLL				
1	66,912	467	23,577	164
2	55,130	84	13,930	21
3	49,022	225	9,236	43
4	23,221	41	12,721	18
5	25,858	50	15,829	30
6	18,791	35	9,245	19
7	17,091	13	16,332	12
8	16,194	24	4,097	6
9	9,450	22	2,889	7
10	3,943	19	7,768	38
11	2,568	9	6,870	24
12	111	1	213	2

other 9 patients showed a higher transformation by soluble PHA, in 6 of these the values were within the range of our controls.

In 5 controls and in 5 CLL patients experiments were performed to enrich B- and T-lymphocytes. The results of the response are seen in figure 4. In controls the percentage of rosette-forming lymphocytes was between 23 and 58%. After centrifugation there was an enrichment of rosette-forming cells in the pellet (56–84%), only few rosettes were detected at the interface (1–5%). In the B-cell rich fraction there was a significantly higher response to insoluble PHA than in the pellet fraction, whereas the pellet had a better response to soluble PHA than the interface fraction.

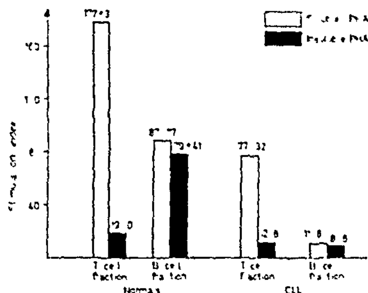


Fig. 4. Response of T- and B-lymphocytes to soluble and sepharose PHA in normals and patients with chronic lymphocytic leukemia.

In patients with CLL we found the known low percentage of rosette forming lymphocytes (5–18%). After Ficoll-centrifugation no rosette forming lymphocytes were seen at the interface, all rosettes were in the pellet. In contrast to normals the B-cell rich fraction showed a minimal response to soluble as well as to insoluble PHA. In the pellet with soluble PHA as mitogen the stimulation index was within the normal range.

Discussion

Mitogens affecting B- or T-lymphocytes to different degrees have been employed for investigating the functional capacity of these cell populations. In the mouse a variety of nonspecific mitogens were tested. PHA and concanavalin A stimulate T-cells, pokeweed mitogen (PWM) and bacterial lipopolysaccharides (LPS) B-cells [4–6]. If PHA is linked to sepharose beads it is also capable of activating B-cells [7]. In the human, PHA acts similarly to the mouse primarily as a T-cell mitogen. PWM stimulates T- and B-lymphocytes. LPS is probably not a very suitable mitogen for human lymphocytes. Mitogens acting mainly on human B-lymphocytes are therefore of considerable interest.

Our experiments show that insoluble PHA almost exclusively in

duced a blastogenic response of the B-cell rich fraction. The suspension with most of the rosette forming cells, on the other hand, gave almost no response to the insoluble mitogen. These results are therefore in agreement with experiments in mice, suggesting a predominant effect of insoluble PHA on B lymphocytes. In CLL a marked blastogenic response to soluble PHA was observed in our T-cell rich suspensions. The blast cell transformation of this fraction was comparable to the normal controls, confirming the results of WYBRAN *et al.* [22]. On the other hand, in the B-cell rich fraction obtained by gradient centrifugation of CLL-lymphocytes, the response to soluble PHA was minimal. A concomitant loss of rosette forming cells and reactivity to soluble PHA was observed. The B-cell nature of lymphocytes in CLL is well-established [8, 10, 16, 17, 20]. There is some evidence of a functionally defective cell population in CLL. Lymphocytes from patients with CLL show a low content of lysosomal enzymes [2], the production of interferon is reduced [14], the receptors for PHA are diminished [13], leukemic B lymphocytes recirculate poorly in comparison to normal B-cells [5]. The fact that leukemic lymphocytes were almost insensitive to insoluble PHA – a mitogen primarily affecting human B lymphocytes – further supports the hypothesis of functionally defective lymphocytes in this disease.

Acknowledgement. We are indebted to Miss F. OBERWASSERLECHNER for skilful technical assistance.

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Acta haemat. 53: 37–43 (1975)

Lymphocytes Devoid of T and B Cell Markers in Chronic Lymphatic Leukemia

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Key Words
Leukemic lymphocytes
Lymphatic leukemia
Lymphocyte immunology

Abstract. Peripheral lymphocytes of 16 patients with chronic lymphatic leukemia were studied for B and T cell markers with peroxidase labelled anti immunoglobulin antisera and complement and spontaneous rosetting techniques. Increased numbers of B cells and normal numbers of T cells were noted. A significant observation was the presence of a large proportion of circulating lymphocytes without the conventional markers for T and B cells. This finding is correlated with clinical features.

Considerable evidence has accumulated to support the idea that bone marrow-derived, bursa-dependent B lymphocytes are increased in the peripheral blood of most patients with chronic lymphatic leukemia (CLL) [1, 12, 14, 17]. The status of T lymphocytes, however, has been the cause of much controversy [7, 10, 15, 17]. Few reports are available in which both T and B lymphocytes have been examined simultaneously in the same patients [15–17]. Analysis of individual cases in these reports suggests that all the lymphocytes circulating in CLL patients cannot be characterized fully. The present work carried out on Indian patients indicates that a significant number of peripheral lymphocytes, at any given time, are devoid of T and B cell markers.

Material and Methods

Sixteen normal controls and 16 patients with CLL were studied. Fifteen of the patients were untreated and one was undergoing therapy. Eleven of them had been diagnosed on routine haematological studies (asymptomatic) and the other 5 patients

[illegible] and lymphocytes [illegible]

[illegible] lymphocytes were [illegible] C_3 [illegible]

[illegible] The cells were [illegible]

[illegible] lymphocytes were also characterized by the [illegible] IgA and IgM were [illegible]

[illegible] IgG [illegible] IgA and IgM [illegible]

The [illegible] were suspended in 0.1 ml of MEM. The cells were [illegible] and [illegible] IgM. The [illegible] lymphocytes were [illegible] with [illegible] The lymphocytes which had been [illegible] The cells were washed [illegible] H_2O_2 [illegible] the cells were [illegible] and IgM [illegible] lymphocytes [illegible]

Results

B lymphocytes were identified [illegible] (EAC rosettes) [illegible]

Table I. Total lymphocyte counts and percentages of E rosettes, EAC rosettes and immunoglobulin-bearing lymphocytes in peripheral blood of CLL patients

Patient	Total lymphocytes $\times 10^3/\mu l$	E rosettes %[1]		EAC rosettes % ± SE	Ig-bearing lymphocytes % ± SE	Unidentified lymphocytes %
		2 h	24 h			
SSC[2]						
a[4]	105.6	–	–	–	15.0 ± 2.5	–
b	207.0	2.0	–	13.0 ± 3.3	14.0 ± 2.4	84.0
PNMP[2]	21.5	0.6	–	27.2 ± 2.0	32.0 ± 2.1	67.4
SPJ[2]						
a[4]	114.0	4.0		41.0 ± 4.9	37.0 ± 2.4	55.0
b	163.0	1.0	–	40.5 ± 3.5	47.0 ± 2.5	52.0
BD[2]	24.4	2.4	–	36.4 ± 2.2	45.2 ± 2.2	52.4
NL[2]	324.9	2.0	2.7	27.1 ± 1.6	–	70.1
DPM	213.8	0.4	–	45.0 ± 2.5	43.6 ± 2.2	54.6
INK						
a[3]	18.0	2.5		–	27.0 ± 2.8	70.5
b	36.6	0.2	–	35.6 ± 2.1	30.3 ± 2.7	64.2
DNDK	15.8	3.2	–	43.0 ± 2.8	44.0 ± 2.2	52.8
BR	53.6	0.6	–	30.4 ± 2.1	31.0 ± 2.1	68.7
KNL	235.2	0.1	–		54.0 ± 2.2	45.9
PVA	62.3	1.2		54.3 ± 2.5	53.7 ± 2.9	44.6
SH	17.6	1.8	2.0	72.6 ± 2.5	69.0 ± 2.6	25.4
HRC	36.8	1.0	2.5	90.0 ± 2.0	40.5 ± 2.2	47.5
RNG	14.8	4.3	5.7	–	35.8 ± 2.0	58.4
R	35.4	2.8	2.6	46.3 ± 2.9	52.0 ± 2.3	45.2
UDG	13.6	6.4	13.7	61.0 ± 2.4	–	32.4
Normals	2.3 ± 0.2 n = 23	47.4 ± 1.5 n = 12	77.3 ± 1.2 n = 12	13.4 ± 0.8 n = 13	13.1 ± 0.6 n = 16	9.8

[1] SEs attached to such small percentages are inaccurate.
[2] Patients in symptomatic group.
[3] Time interval between tests = 3 weeks.
[4] Time interval between tests = 2 months.

cases there was a good correlation of the percentage of lymphocytes identified as B cells by the 2 techniques (table I) [11]. Peroxidase-labelled immunoglobulin determinants appeared as brown specks or dots on the membrane of B lymphocytes. They were distributed randomly on the

Table II Total lymphocyte counts and percentages of E rosettes, EAC rosettes and immunoglobulin-bearing lymphocytes in peripheral blood of 3 patients followed up after 3–12 months

Patient	Interval since test in table I months	Total lymphocytes $\times 10^3/\mu l$	E rosettes % 2 h	E rosettes % 24 h	EAC rosettes % ± SE	Ig bearing lymphocytes % ± SE	Unidentified lymphocytes %
INK	3	27.46	1.0	8.7	62.5 ± 2.4	–	29.8
INK	10	29.60	4.9	6.6	79.6 ± 2.3	77.3 ± 2.4	13.8
PNM[1]	5	27.74	2.0	3.0	74.0 ± 3.1	63.2 ± 2.4	23.0
PNM[1]	12	15.25	3.3	11.7	62.6 ± 2.8	59.5 ± 3.5	25.7
DPM	11	161.68	3.6	5.6	80.6 ± 1.8	76.5 ± 3.0	13.8

1 Patient in symptomatic group

membrane. Polar localization was seen in only 1% of the cells, even when the incubations were carried out at room temperature.

The total lymphocyte counts in the CLL patients varied from 14 to $325 \times 10^3/\mu l$ (table I). B lymphocytes were significantly increased in 15 of the 16 patients (table I). The normal range of B lymphocytes was 9–18%, or $0.086–1.01 \times 10^3/\mu l$ in absolute terms. In 15 of the CLL patients studied, the percentage of B cells was increased to 27–72.6% with absolute numbers of $5–127 \times 10^3/\mu l$ (table I). SSC was an exception with 14% B lymphocytes as checked on 2 separate occasions. The percentage of cells bearing IgM and IgA markers was in the range of 3–6 and 1–3% respectively.

T lymphocytes were identified by their ability to form spontaneous rosettes with sheep erythrocytes (E rosettes) after 2 and 24 h of incubation at 4 °C. In control subjects, an average of 47.4% of peripheral lymphocytes formed spontaneous rosettes at 2 h, and by 24 h this percentage increased to 77.3% (table I). In CLL patients, the percentage of 2 hour T cells was markedly low (table I). A mild increase in percentage was noted after 24 h of incubation at 4 °C. However, the absolute T cell numbers in the circulation of these patients were within the normal range.

There was no relationship between the total leucocyte counts and the percentage of circulating B and T cells. It is significant that in 14 of the 16 patients 40% or more of circulating lymphocytes could not be identified by the methods employed (tables I). By morphological criteria on Giemsa stained smears, these cells were not different from those identified as B and T lymphocytes. It is noted that in 4 patients in the sympto-

matic group the circulating B cells were 40% and below, whereas the non-identifiable cells were 50% and more Only 2 (INK and BR) of the 11 asymptomatic patients had very high levels of non identifiable cells (64–70% table I)

Follow-up studies on 3 patients 3–12 months after the first tests showed that in all 3 cases lymphocytes bearing B cell markers had markedly increased while T cell numbers remained in the normal range The percentage of unidentifiable cells was reduced to less than 30% (table II)

Discussion

This study was designed to investigate the nature of the lymphocytes circulating in patients with chronic lymphatic leukemia The enzyme marker peroxidase was used in the demonstration of Ig bearing B cells With a view to studying the total population of B lymphocytes in circulation, cells bearing both surface Ig and complement receptors were measured These studies in both the normal subjects and patients indicate a good correlation between the 2 methods The range of B cells in the controls (9–18%) also corresponds well with earlier reports [8, 12, 14]

The present work indicates that B cells are increased severalfold in patients with CLL. The percentage of B cells in this group of patients studied does not fall in the extremely high or low ranges reported by some workers [12 13] Our data is similar to that of Piscus *et al* [14] who reported a B cell increase to 50 ± 25%. It is evident, however, that in terms of absolute numbers our patients have very high numbers of circulating B cells

The percentage of circulating T lymphocytes forming spontaneous rosettes is markedly low (1–4%) in all the patients of CLL. However, in view of the large error inherent in reading a small proportion of positive cells all patients appear to have near normal absolute numbers of circulating T cells Therefore the marked reduction in the percentage of T lymphocytes noted in CLL appears to be due to the dilution effect of high numbers of other cells This is in accordance with the data of Wybran *et al* [17] who demonstrated normal transformation with phytohaemagglutin when the dilution effect of the B cells was removed

The striking feature in some of our patients is the presence of 40% or more cells which are not identifiable as either B or T cells. Incubation of lymphocytes with sheep erythrocytes for 24 h at 4°C significantly in-

creases the E rosettes in the normal and thus reduces the unidentifiable cells to a minimum. However, in the CLL patients, even though there is an increase in E rosettes at 24 h, this does not contribute significantly to the percentage of identifiable cells. The population of cells without surface markers, therefore, remains high. Similar results have also been reported by McLAUGHLIN *et al.* [9]. The identity of this population of cells in CLL is not clear. The possibility exists that these may be abnormal T cells without receptors for sheep erythrocytes. However, in view of the fact that B cells are markedly increased and T cells are in normal numbers, it seems likely that these cells belong to the B cell category where immunoglobulin and complement receptors are not fully expressed. This view is supported by the fact that in 3 follow up studies the percentage of B lymphocytes increased markedly with a decrease in unidentifiable cells, while T cells remained in normal numbers.

CLL may represent a spectrum of disease which corresponds to the degree of abnormality in the B cells. The formation of surface Ig may be a property acquired during cell maturation. One of our patients (SSC) who had low levels of B cells, was severely ill and died within 6 weeks of the second evaluation. It may also be emphasized that higher percentages of B cells were noted in the asymptomatic group. Lower numbers of unidentifiable cells were also a feature of the asymptomatic patients. The number of lymphocytes attaining the Ig markers may reflect a better clinical state of the patient. From our preliminary data, it appears that the identification of both B and T cells has a prognostic value in CLL. Numbers of circulating lymphocytes without surface markers may form an important criterion for assessing the clinical form of the disease.

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Acta haemat. 61: 44-48 (1979)

Dissociation between Factor VIII (Activity and Antigen) and Ristocetin-Induced Platelet Aggregation in von Willebrand's Disease

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Abstract. Previously we showed that von Willebrand patients could be separated into 2 groups on the basis of ristocetin-induced platelet aggregation and factor VIII (antigen and activity). Ristocetin-induced platelet aggregation and factor VIII exhibited a positive correlation. This paper deals with 5 out of 31 examined von Willebrand patients in which ristocetin-induced platelet aggregation was not in correlation either with factor VIII activity or antigen. The importance of these findings is discussed.

Key Words
Coagulation disorders
Factor VIII antigen
Platelet aggregation
Ristocetin
Von Willebrand's disease

Recently it has been shown that ristocetin-induced platelet aggregation is impaired in several cases of von Willebrand's disease and that the abnormality may be corrected by addition of normal or haemophilic plasma [1, 5, 8].

Weiss *et al.* [9] found a positive correlation between factor VIII activity, factor VIII antigen and the magnitude of platelet-induced aggregation by ristocetin in von Willebrand patients. They suggested that factor VIII refers to a molecule on which 3 activities are located: the antihaemophilic factor procoagulant activity (AHF), the antigen (AGN) identified by rabbit anti-VIII antiserum and the factor necessary for ristocetin induced platelet aggregation (von Willebrand factor, vWF).

AHF, AGN and vWF have been found proportionally decreased in all von Willebrand patients examined by Weiss *et al.* [9].

We report here some von Willebrand patients exhibiting a dissociation of the properties of the factor VIII molecule.

Material and Methods

31 patients with von Willebrand's disease were examined. Criteria for diagnosis were as follows: prolonged bleeding time assessed using Ivy's method, decreased factor VIII activity measured on freshly drawn plasma by a one-stage activated partial thromboplastin time method, using haemophilic plasma as substrate, decreased platelet adhesiveness, performed according to SALZMAN [7], history of haemorrhage and demonstration of family autosomal dominant disease. Most of the patients have been the subjects of previous studies [2].

Plasma factor VIII antigen was determined by Laurell's technique using Behringwerke antiserum, as previously described [3].

Platelet rich plasma (PRP) was prepared by centrifuging citrated blood (1 part citrate to 9 parts blood) for 15 min at 600 rpm. Platelet poor plasma (PPP) was obtained by centrifuging PRP at 5 000 rpm for 30 min. After an initial platelet count, PRP was diluted with PPP to give a platelet concentration of 300 000/mm³. 1 ml of PRP with known platelet number was distributed into silicone-coated glass test tubes.

Platelet aggregation was measured turbidometrically in Mustard's aggregometer attached to a Bausch Lomb recorder. Before carrying out the determinations, zero transmission was set by adjusting the recorder tracer with PRP. PPP was used to fix 100%. Platelet aggregation was expressed as the percent change in optical density of PRP after addition of ristocetin.

Ristocetin sulphate was supplied by Lundbeck, Copenhagen, and dissolved in normal saline to produce a final concentration in PRP of 1.0 mg/ml.

Platelet count was performed according to PALUMBO and DINI [6].

Results

Factor VIII antigen was normal in 10 patients (our normal range being from 50 to 160%). Five patients showed normal ristocetin induced platelet aggregation and 1 just at the lower limit of normal (our normality being greater than 50%).

Figure 1 gives the relationship between ristocetin induced platelet aggregation and factor VIII antigen. The greater part of patients presented a positive correlation between ristocetin aggregation and factor VIII antigen while 5 patients presented a low degree of ristocetin aggregation despite of normal amount of factor VIII antigen.

Mean and standard deviation of coagulation values are given in table I. Groups A and B refer to the patients exhibiting a positive correlation between ristocetin aggregation and factor VIII antigen. Group C includes the patients with poor ristocetin platelet aggregation and normal factor VIII antigen. The results of statistical analysis are presented in table II.

Table I. Coagulation values in the 31 von Willebrand patients

Groups	Bleeding time min (normal 3-8) mean ± SD	Platelet adhesiveness, % (normal 25-50) mean ± SD	Factor VIII activity, % (normal 60-140) mean ± SD	Factor VIII antigen, % (normal 50-140) mean ± SD	Ristocetin aggregation, % (normal 50-70) mean ± SD
Group A, n = 21	10.42 ± 6.39	9.57 ± 11.45	15.71 ± 9.08	11.52 ± 9.17	9.00 ± 10.41
Group B, n = 5	24.20 ± 14.80	3.80 ± 6.49	49.00 ± 22.47	89.40 ± 17.51	72.00 ± 5.70
Group C, n = 5	20.00 ± 13.90	8.00 ± 9.08	35.60 ± 4.39	95.60 ± 25.34	7.00

The patients were separated into 3 groups (A, B, C) according to the relationship between ristocetin-induced platelet aggregation and factor VIII antigen.

Table II. Relations between ristocetin-induced platelet aggregation and factor VIII (activity and antigen), platelet adhesiveness, and bleeding time (r and p from linear regression analysis)

	Number of pairs	r	p
Ristocetin aggregation and factor VIII activity	31	0.71	< 0.001
Ristocetin aggregation and factor VIII antigen	31	0.53	< 0.01
Ristocetin aggregation and platelet adhesiveness	31	-0.18	n.s.
Ristocetin aggregation and bleeding time	31	0.33	n.s.

n.s. = Not significant

Discussion

In a previous paper [1] we showed that von Willebrand patients could be subdivided into 2 groups on the basis of ristocetin aggregation. The values of ristocetin aggregation were in correlation both with factor VIII antigen and activity, so that one may conclude that ristocetin aggregation might be a measure of entire factor VIII molecule. But, in a wider group of patients, we recognized 5 out of 31 cases, in which ristocetin aggregation was not in correlation either with factor VIII antigen or activity. The 5 patients exhibited values of ristocetin aggregation varying between 5 and 10%, while the factor VIII protein ranged between 50 and 130%. Four of them showed reduced factor VIII activity but normal amount of factor VIII antigen. The other patient had normal factor VIII activity

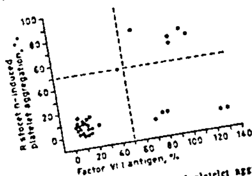


Fig 1 Relationship between ristocetin-induced platelet aggregation and factor VIII antigen Dashed lines represent the lower limits of normal

and antigen These findings are of interest as they suggest that there are some von Willebrand patients with reduced factor VIII activity but with normal amounts of factor VIII antigen This observation has been reported also by HOLMBERG and NILSSON [4] Moreover, one may assume that ristocetin-induced platelet aggregation depends on a factor different from factor VIII antigen and activity, probably the vWF

Therefore, von Willebrand patients may be separated into 3 groups on the basis of ristocetin aggregation and factor VIII antigen (groups A, B, C in table I) Group A, characterized by low degree of ristocetin aggregation and low plasma content of factor VIII antigen, includes the majority of cases, while group B, normal ristocetin aggregation and normal factor VIII antigen, and C, poor ristocetin aggregation and normal factor VIII antigen are much less frequent

According to WEISS *et al* [8, 9] the defect in von Willebrand's disease may be due to a decreased synthesis of the entire factor VIII molecule, which results in a proportional decrease in factor VIII antigen, activity and vWF These authors also suggest the possibility of an additional abnormality in the site of the molecule, that determines the activity of vWF The cases here presented, belonging to group C, seem to confirm this possibility

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Acta haemat. 53: 49-55 (1975)

Un cas de myelose aiguë atypique: une leucemie ARN

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Abstract. In a 53 year-old female patient a tumor localized in the nasopharyngeal cavity together with hematologic features of acute paramyeloblastic leukemia were observed. More than 70% blast cells contained giant intracytoplasmic inclusions which have been found to be strongly pyroninophilic. The electron microscopic study revealed big agglomerations of pseudofibrillar structures of the ribosomal RNA inside of cytoplasma. The possibility of a new nosological entity should be envisaged.

Key Words
Acute myeloid leukemia
Cellular inclusions
Cytochemistry
Electron-microscopy
RNA-leukemia
Tumoral leukemia

Nous rapportons l'observation d'un cas de leucémie aiguë à début tumoral localisée dans la cavité naso-pharyngienne. Chez ce sujet nous avons observé dans le cytoplasme des blastes des inclusions géantes qui ont été identifiées comme étant de l'ARN ribosomal. La morphologie cellulaire ainsi que la symptomatologie clinique montrent donc des particularités intéressantes.

Données cliniques et morphologiques

Mme O. P., âgée de 53 ans, est admise à l'Institut d'Oncologie le 22 octobre 1973 pour une tuméfaction de la région buccale gauche, surélevée, dyspnée et anémie. La température est normale, le pouls 96/min rythmique. La marge inférieure du foie est palpable 2 cm au-dessous de l'arc costal, consistance ferme mais souple, indolore. Toute la région buccale gauche est gonflée, accompagnée d'une adénopathie cervicale sous-angulaire, sous-mandibulaire et sus-claviculaire gauche. La narine gauche est gonflée et cyanotique. Une tumeur envahit totalement la voûte naso-pharyngienne; sa surface est pâle et présente quelques fragments rouges, nécrosants. L'examen histologique de la biopsie montre une infiltration microcellulaire probablement néoplasique; elle ne possède

aucun des traits caractéristiques du granulome de Wegener L'examen radiologique montre une opacification partielle de la cavité maxillaire droite sans altérations osseuses La radiographie du thorax est normale

Examens complémentaires du 23 octobre 1973 Hématies, 2230000, hémoglobine, 7,9 g/100 ml Leucocytes, 14900 dont 90% de blastes Plus de 70% de ces blastes possèdent dans le cytoplasme des inclusions arrondies de 1,0 à 3,0 μm de diamètre, qui se colorent par la méthode de May Gruenwald-Giemsa d'une façon qui les fait ressembler à la chromatine Les contours des noyaux sont parfois transparents, avec une superposition qui rappelle le pliage de la matière nucléaire (fig 1a) Outre les blastes, on observe aussi 6% de lymphocytes, 2% de polynucléaires, 1% d'éosinophiles et 1% de monocytes Les hématies montrent une anisocytose et une poïkilocytose Plaquettes, 210000, hématocrite, 22%, vitesse de sédimentation, 35/h La moelle osseuse sternale est infiltrée totalement par les blastes décrits dans le sang (fig 1b)

Réactions cytochimiques Coloration à la peroxydase (méthode de Sato), positive (fig 2a), coloration au «Sudan black B», négative, coloration au «oil red», négative, coloration au PAS, positive, diffuse, coloration par la méthode de Feulgen, négative dans les inclusions (fig 2b), coloration à la phosphatase acide (méthode de Gomori) négative, à la coloration par la méthode de Brachet, les inclusions s'avèrent fortement pyroninophiles (fig 2c) Au vu de ces résultats, le diagnostic d'une leucémie aigue myéloide ou myélo-monocytaire à paramyéloblastes fut établi

Examen de l'ultrastructure Le sang héparinisé fut centrifugé à 1000 t/min pendant 5 min La couche des leucocytes fut préfixée dans une solution tampon d'aldéhyde glutarique Les préparations furent rincées puis fixées dans une solution tampon de tétroxyde d'osmium Après déshydratation, les petits fragments furent fixés dans de l'épon Les coupes ultra-fines furent contrastées dans de l'acétate d'uranyle et du citrate de plomb et examinées au microscope électronique TESLA BS 513

Résultats Les nucléoles, qui ont passé inaperçus au microscope optique, apparaissent très nettement Ils ont des dimensions importantes et leurs contours sont irréguliers (fig 3a) Les granulations primaires sont rares (fig 3) Nous n'avons trouvé aucune fusion de ces granulations, ni formation des corps d'Auer Le nombre de ribosomes est important, très fréquemment ils peuvent être rencontrés à l'intérieur des structures filamenteuses en anneaux, qui correspondent aux «zones en croissant» décrites par BESSIS *et al* [2, 3] (fig 3) Les mitochondries sont nombreuses et parfois géantes (fig 3e) On peut les trouver aussi enfermées dans les «zones» (fig 3a–d) Quelques zones contiennent des *fragments du réseau endoplasmique lisse très ramifié et condensé (fig 3e, f) On note* aussi de nombreuses vacuoles et des grains de glycogène dans le cytoplasme (fig 3)

En raison de l'apparition d'une occlusion respiratoire presque complète, nous avons irradié la tumeur par une dose totale de 480 R, répartie en 3 doses de 160 R, espacées de 3 jours Immédiatement avant et après l'irradiation, la malade a reçu trois transfusions à 250 ml de sang frais L'état local s'améliore, la dyspnée diminue Cependant, en quelques jours une otite purulente apparaît au côté droit Un nouvel examen du sang révèle une leucocytose (12900 dont 80% de paramyéloblastes, 13% de polynucléaires, 4% de monocytes, 2% de lymphocytes et 1% d'eosinophiles Hématies, 2810000, hémoglobine, 8,8 g/100 ml, vitesse de sédimentation, 48/h

Malgré une antibiothérapie et une corticothérapie, l'état de la malade continue à s'aggraver Le 30 novembre 1973, les douleurs abdominales accompagnées d'anurie et un



Fig. 1. Frottis. May-Gruenwald Giemsa. *a* Sang, inclusions intracytoplasmiques et plage de la matière nucléaire dans les blastes. *b* Moelle osseuse: groupe de blastes montrant des inclusions. × 900.

Fig. 2. Frottis sanguine. *a* Coloration à la peroxydase par la méthode de Sato: les blastes montrent une réaction positive. *b* Coloration par la méthode de Feulgen: les inclusions ne se colorent pas (flèches). *c* Coloration par la méthode de Brachet, les mêmes structures s'avèrent fortement pyroninophiles (flèche). × 900.

œdème aigu des poumons survient. La malade meurt le même jour moins de 6 semaines après son hospitalisation.

Cette malade de 43 ans avait donc présenté une affection aiguë d'évolution rapidement mortelle caractérisée cliniquement par une invasion néoplasique de la cavité naso-



Fig 3 Sang, microscopie électronique *a* Un blaste contenant un nucléole grand et irrégulier, ainsi que deux «zones en croissant» (flèches), il convient de noter le grand nombre de vacuoles, de ribosomes, de mitochondries de grande taille et les grains du glycogène × 11700. *b* Dans la même cellule on voit enfermés dans les «zones» de très nombreux

pharyngienne, des adénopathies superficielles et une hépatomégalie. Du point de vue hématologique le diagnostic d'une myélose aiguë à paramyéloblastes était évident.

Diagnostic anatomique. L'autopsie pratiquée le 1er décembre 1973 montre un œdème pulmonaire et cérébral important, un foie hypertrophié, des tuméfactions ganglionnaires superficielles et médiastinales, un envahissement bilatéral par des masses tumorales de toute la cavité naso-pharyngienne et maxillaire, ainsi qu'une otite purulente bilatérale. Histologiquement, on retrouve dans les coupes de tous les organes, à l'exception du cœur, des muscles striés et du tissu sous-cutané, une prolifération des cellules mononucléaires rondes, dont l'aspect est uniforme et correspond à celui des cellules qui constituent le contenu de la cavité sternale.

Commentaires

Sur le plan clinique, la localisation primaire suggère le groupe entier de maladies que les auteurs anglo-saxons appellent «lethal midline granuloma». Ce terme vaut pour toutes les lésions évolutives et destructives localisées dans la région naso-pharyngienne, y compris les lympho-sarcomes, les réticulo-sarcomes et le granulome de Wegener [11, 16]. Ce dernier désordre peut être exclu, puisque les altérations typiques, c'est-à-dire une vasculite nécrotisante généralisée, accompagnée d'une néphrite et d'une réaction allergo-inflammatoire [19], ne furent pas observées. L'origine lymphatique de la tumeur doit être rejetée en vue de la réaction positive à la peroxydase.

Notre cas ressemble aux réticulo-histiocyto-sarcomes rapportés par DE FARIA *et al.* [8] et à ceux de HULTBERG *et al.* [14]. Toutefois, des blastes sanguins contenants des inclusions ne furent pas décrits par ces auteurs. Il reste alors à établir s'il s'agit d'une tumeur de provenance myélomonocytoïde qui finit avec une dissémination ultérieure hématogène, ou bien d'une leucémie aiguë à début tumoral [7]. L'effet fatal de l'irradiation plaide, à notre avis, en faveur d'une leucémie.

Sur le plan cytologique, les inclusions font penser au syndrome de Chediak-Higashi-Steinbrick [6, 17]. Pourtant, l'âge de notre malade, les manifestations cliniques et les données de la microscopie rendent ce diagnostic inacceptable [1, 4, 9, 20]. Les inclusions ne correspondent pas davantage

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...somes et mitochondries ... ×31400. *c* Un blaste contenant une «zone en croissant» (flèches). À noter l'absence de granulations primaires. ×11700. *d* La même «zone» remplie par des mitochondries et des ribosomes. ×23400. *e* Un autre blaste avec une «zone en croissant» (flèche). ×11700. *f* La «zone» est remplie par des ribosomes et le réseau endoplasmique lisse; ... la structure est ramifiée et fortement condensée. ×23400

aux bâtonnets d'Auer, qui sont des agglomérations de granules azurophiles, caractérisées par une structure organisée, lamellaire ou semi-cristalline et qui montrent une réaction positive à la phosphatase acide [5, 10, 12, 16, 18] Les corpuscules de Dœhle n'apparaissent que dans les éléments sanguins mûrs Leur ultrastructure est caractérisée par les lamelles du réseau endoplasmique raboteux, dilatées et bordées de ribosomes [3] Par contre, nous avons trouvé nos inclusions dans les cellules indifférenciées

Les structures que nous venons de décrire montrent une pyroninophilie évidente Au microscope électronique, on voit les amas de ribosomes, très fréquemment enfermés dans les «zones en croissant» Ces zones, composées de fibrilles disposées concentriquement et formant des sphères ou des cylindres creux, ne doivent apparaître que dans les leucémies myéloblastiques et monocytaires [5, 13] Ce sont, surtout, ces «zones», remplies par l'ARN ribosomal, qui sont responsables de l'aspect bizarre des cellules observé dans la coloration panoptique et dans celle de Brachet Il faut accentuer le pourcentage extraordinairement élevé de cellules qui montrent cette anomalie, ainsi qu'une localisation inhabituelle de ribosomes Ces altérations témoignent d'une atteinte grave du métabolisme nucléoprotidique chez notre sujet

Si l'on tient compte, de plus, des manifestations cliniques atypiques, ces observations morphologiques semblent donner à ce cas un trait de nouveauté C'est pourquoi nous considérons l'observation digne d'être rapportée dans le but d'attirer l'attention sur ce désordre qui – peut-être – constitue une entité nosologique nouvelle Nous voudrions proposer le terme de «leucémie ARN»

Remerciement Nous remercions Mme Dr KRZYŻOWSKA GRUCA d'avoir exécuté et interprété les examens au microscope électronique

Résumé

Le cas d une malade, âgée de 53 ans, que nous venons de rapporter est caractérisé par l'apparition d une tumeur localisée dans la cavité naso pharyngienne, alors qu'au point de vue hématologique il possède tous les traits typiques d une leucémie aiguë à paramyéloblastes Dans plus que 70% de ces blastes on trouve des inclusions intracytoplasmiques géantes, fortement pyroninophiles La microscopie électronique y révèle des amas de ribosomes enfermés dans les structures filamenteuses en anneaux, les «zones en croissant» Il peut s'agir d une entité nosologique nouvelle

Leucémie ARN

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Acta haemat 53 56–59 (1975)

Haemoglobin Stanleyville II (α 78 [EF 7] Asn → Lys) Found in France

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Abstract Haemoglobin Stanleyville II has been found several times in individuals from Central Africa This paper now reports its discovery in Alsace in a family of long standing local ancestry

Key Words
Haemoglobinopathies
Hb Stanleyville II
Mucoviscidosis

In the Centre de Transfusion Sanguine de Strasbourg, an 8-year-old girl, who was affected with Mucoviscidosis, was found to possess an abnormal haemoglobin This haemoglobin, representing 24% of the total, had a mobility similar to Hb S on electrophoresis at pH 9 2, but at pH 6 2 similar to that of Hb A The mother and 5 of the 7 siblings possessed this abnormal haemoglobin Only 2 of these 5 children had all the symptoms of Mucoviscidosis The other 3 were less seriously affected and gave only abnormal sweat tests

Hb Stanleyville II was first found in 2 families in the north east of the Congo, which is inhabited by a population of mixed Bantu-Nilotic origin [1] It was then discovered in a third family of Nilotic stock from the West Nile region of Uganda [2] and in a young nun, also of Nilotic stock, from north east Zaire [3] Finally, Hb Stanleyville II has been reported in a family of Sudanese ancestry of the Ngbandi tribe in the north of the Republic of Zaire [4] In several instances the haemoglobin was found in association with Hb S The family now being reported comes from Alsace and has been living there for many generations

Materials and Methods

Haemolysates were fractionated using column chromatography on DEAE Sephadex according to the method of HUISMAN and DOZY [5] The abnormal haemo

globin fraction was concentrated and purified by repeated electrophoresis on filter paper in Tris EDTA borate buffer at pH 8.9 [6]. Globin was prepared by precipitation (1.5% v/v of concentrated HCl in acetone) at 0 °C [7] and washed 3 times in acetone at 0 °C.

Tryptic digestion was carried out at a concentration of 10 mg polypeptide/0.2 mg TPCK treated trypsin/ml ammonium bicarbonate buffer (pH 8.5) at 37 °C for 2 h. (Trypsin treated with L-(1 tosylamido-2 phenyl) ethyl chloromethyl ketone (TPCK) to inhibit α-chymotryptic activity was obtained from Worthington Biochemical Corp., Freehold, N.J., USA.) The soluble tryptic peptides were fingerprinted using high voltage electrophoresis at pH 6.4 followed by ascending chromatography at right angles [8]. The fingerprints were stained with ninhydrin (0.2% in acetone) and with reagents specific for methionine, histidine, arginine, tyrosine and tryptophan [9].

To facilitate isolation of the abnormal peptides the α- and β-polypeptide chains were separated on a column of Whatman CM 23 using a gradient of phosphate buffer in 8 M urea at pH 6.7 (0.01 M to 0.10 M to Na) [10]. The abnormal α-chains were eluted at a higher sodium ion concentration than normal α-chains. The eluate containing the abnormal α-chains was desalted by dialysis against at least 5 changes of 6 litres of 0.5% formic acid at 6 °C and lyophilised. The abnormal tryptic peptides were isolated by preparative fingerprinting of the abnormal α-chain globin [11] and, where necessary, purified by further electrophoresis at either pH 3.5 in a Michl tank or pH 9.0 on a flat bed electrophoresis plate. The purified abnormal peptides were eluted with constant boiling HCl, hydrolysed in sealed capillary tubes at 110 °C for 18–24 h, dried over NaOH *in vacuo* and subsequently analysed on a Locarte Amino Acid Analyser [12].

Results

Figure 1 shows a fingerprint of the soluble tryptic peptides obtained from the abnormal haemoglobin. It differed from that of Hb A by the absence of the electrically neutral peptide αTpIX (α62–90) and the positively charged peptide αTpVIII–IX (α61–90) (1 and 5 in fig. 1), both of which contain methionine and histidine, and by the presence of 3 new peptides (2, 3 and 4 in fig. 1). One of these new peptides gave a positive reaction for histidine (2 in fig. 1) and the other 2 for methionine (3 and 4 in fig. 1). The area 2 (fig. 1) was separated into 2 peptides by electrophoresis at pH 3.5 (see Materials and Methods), αTpIII and a new peptide which gave an amino acid composition identical with residues 79–90 of the α^{X}-chain (table I). This new peptide was thus called αTpIXb. The area 3 (fig. 1) was shown by electrophoresis at pH 9.0 to consist of 2 peptides, αTpV and a new peptide possessing the amino acid residues 62–78 of the α^{X}-chain except that one aspartyl residue



Fig. 1. Fingerprint of Hb Stanleyville II. 1 = Peptide αTpIX missing; 2 = new αTpIXb (histidine-positive) + αTpIII; 3 = new αTpIXa (menthionine histidine positive) + αTpIV; 4 = new αTpVIII-IXa (methionine histidine positive) + βTpI; 5 = peptide αTpVIII-IX missing. The peptides were subsequently further separated (see text).

Table I. The amino acid composition (molar ratios) of αTpIXa and αTpIXb from Hb Stanleyville II

Amino acid	Hb Stanleyville II		Hb A
	αTpIXa	αTpIXb	αTpIX
ASP	3.8	1.2	6
THR	1.0	–	1
SER	–	1.9	2
PRO	0.7	–	1
ALA	4.2	3.0	7
VAL	3.0	–	3
MET	0.8	–	1
LEU	1.0	3.0	4
HIS	1.1	1.9	3
LYS	1.0	1.1	1
Yield, nmol/residue	6.6	8.9	

was missing and had been replaced by one lysine residue (table I). This new peptide was called αTpIXa. It was assumed, because of the specificity of trypsin, that lysine was in the C-terminal position of this peptide, where an asparagine is present in the α-chain of Hb A.

It was concluded that the mutation was asparagine 78 (EF7) → lysine and that the variant was *Hb Stanleyville II*.

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Acta haemat 53 60–64 (1975)

A Case of Homozygous Haemoglobin Lepore$_{Boston}$ in Iran

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Abstract Homozygous haemoglobin Lepore$_{Boston}$ disease is described in an Iranian 16-year old boy presenting the clinical manifestations of β thalassaemia major The parents were related (cousins) and both carriers of Lepore trait It seems that the symptoms are milder in this case than what reported before

Key Words
Hb Lepore
Haemoglobinopathies
Thalassaemia

Lepore haemoglobins are the result of unequal crossing-over between structural genes coding for δ- and β-polypeptide chains of human haemoglobin during meiosis The abnormal polypeptide chain $\delta\beta$ produced after this crossing-over, has part of the δ chain at its N-terminus and the remaining is similar to the β-chain [1] The point of the fusion of two polypeptide chains, and the proportion of the δ- and β-chains varies in different types of Lepore haemoglobins In haemoglobin Lepore$_{Boston}$, the point of the fusion of two polypeptide chains is somewhere between residue 87 of the δ-chain and 116 of the β-chain [7]

Haemoglobin Lepore$_{Boston}$ has been reported before in heterozygous, *homozygous and double heterozygous states in association with* α-thalassaemia [3, 9–11] In this article, we describe a case of homozygous haemoglobin Lepore$_{Boston}$ in an Iranian family presenting the clinical manifestations of β-thalassaemia major The disease looks to be milder in this case than the cases reported by FESSAS *et al* [3]

Case History

The propositus was a 16 year old boy admitted to the children s hospital with preliminary diagnosis of Cooley s anaemia He has been splenectomised 8 years

ago and has the characteristic mongoloid facies, hepatomegalia and growth retardation. RBC 3.4×10^6 mm³, WBC 12400 mm³, haematocrit 30%, haemoglobin 10.2 g/100 ml, reticulocytes 9%, MCV 87 μm³, MCH 30 pg, MCHC 34%. Red cell morphology: hypochromia ++, anisocytosis ++, poikilocytosis ++, microcytosis +++.

The parents of the propositus were cousins. The father, aged 57, presented a mild anaemia: Hb 11.8 g/100 ml, RBC 4.2×10^6 mm³, haematocrit 34%, reticulocytes 3%; red cells were hypochromic, anisocytosis, poikilocytosis and target cells were present. Mother, aged 43, was anaemic: RBC 3.7×10^6 mm³, Hb 11.4 g/100 ml, haematocrit 32%, reticulocytes 3%. Red cells presented hypochromia, anisocytosis, poikilocytosis, microcytosis and target cells. The propositus has two married sisters not available for study and there is the history of a died child at 6 from unknown reason.

Haemolysates were prepared by washing the red cells 3 times with 0.9% saline and lysed by the addition of 1 vol of distilled water and 0.5 vol of carbontetrachloride to the packed red cells followed by centrifugation. Fetal haemoglobin was estimated by 1 min alkali-denaturation of SINGER *et al.* [14], to be 73.5% of total haemoglobin. Heat denaturation was negative according to GRIMES *et al.* [5].

Electrophoresis of the haemolysates at alkaline pH on cellulose acetate [4], and in starch gel pH 8.6 revealed the presence of fetal haemoglobin (HbF) and another slow moving band in the same position of HbS, with complete absence of HbA and HbA_2. Examination of the parent's haemolysates showed the same slow moving haemoglobin band in addition to HbA. There was no fetal haemoglobin found in the parents haemolysates, but HbA_2 was present. The proportion of the abnormal haemoglobin fraction was 23% of total haemoglobins as estimated by elution of haemoglobin bands after electrophoresis on cellulose acetate [8].

Separation of α- and β-polypeptide chains of haemoglobins by electrophoresis of the haemolysates on cellulose acetate according to UEDA and SCHNEIDER [15] using buffers containing 6 M urea and 0.05 M 2-mercaptoethanol, as well as treatment of haemolysates with p-hydroxymercuribenzoate (PMB) followed by starch gel electrophoresis pH 8.6 [13], revealed that the abnormality existed in the β-chain (non-α-chain). With such a low proportion of the abnormal fraction, without being unstable and the entirely absence of HbA and HbA_2, and large amount of fetal haemoglobin in the propositus, homozygous haemoglobin Lepore was suspected.

The abnormal haemoglobin was purified by column chromatography of the haemolysates on DEAE-cellulose (Whatman DE 52), using Tris-HCl buffer pH 8, 0.05 M [6], and the protein was concentrated in vacuo. Globin was prepared by treatment of purified haemoglobin with 2% HCl in acetone at −20 °C, the precipitates were washed with cold acetone and freeze dried.

Peptide chains of the globin were prepared by CMC column chromatography of the globin in phosphate buffers at pH 6.8, containing 8 M urea and 0.05 2-mercaptoethanol according to CLEGG *et al.* [2]. The chains were aminoethylated and tryptic digestion and fingerprinting of S-aminoethylated chains were carried out [2]. Figure 1 shows the fingerprints of the S-aminoethylated non-α-chain of the abnormal haemoglobin. It can be seen that the peptide β-Tp3 is missing from its usual place (1) and has shifted towards the cathode (2).

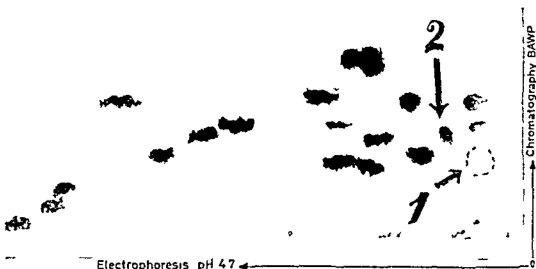


Fig 1 Fingerprints of the S aminoethylated non α chain of Hb Lepore 1 = Peptide β Tp3 is missing from its usual place 2 = new peptide with the same amino acid composition of δ-Tp3

Peptides T3 and T5 were eluted from four preparative fingerprints of the non α chain of the abnormal haemoglobin with 6 N HCl and hydrolysed at 110 °C for 20 h in micropipettes The amino acid composition of each peptide was determined on an automatic amino acid analyser (Hitachi KLA 5) The results indicate the same amino acid composition found in the respective tryptic peptides of the δ chain namely δ Tp3 and δ Tp5 An alanine was found in δ Tp3 instead of the glutamic acid in the β Tp3 and a serine residue was found in δ Tp5 instead of threonine in the peptide β Tp5 This serine at residue 50 is a good index of the presence of haemoglobin $Lepore_{Boston}$

Discussion

The clinical manifestations in the propositus, and the presence of the large amount of fetal haemoglobin in the haemolysates, mimics β-thalassaemia major which is frequently found among Iranians [12] The demonstration of an abnormal haemoglobin with Lepore characteristics in the haemolysates of the propositus and his parents, the complete absence of HbA and HbA_2, in the propositus and the investigation of the abnormal haemoglobin, made the diagnosis of homozygous haemoglobin $Lepore_{Boston}$ Homozygous haemoglobin Lepore has been reported before by FESSAS *et al* [3], NEEB *et al* [9], and QUATTRIN *et al* [10] In

our patient, the disease looks to be milder than the cases reported by Fessas *et al.* [3], having haemoglobin levels between 5.6 to 9.1 g/100 ml and RBC 2–3 millions/mm³.

Acknowledgement. This work is supported by Research Grants from The University of Tehran and the Ministry of Science and Higher Education of Iran.

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Acta haemat. 53: 65–74 (1975)

Some Aspects of Erythropoietic Cell Proliferation in Erythroleukaemia

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Abstract. Cytophotometric autoradiographic investigations on erythropoiesis in erythroleukaemia indicate: (1) the decreased labelling index of erythroblasts is explained by an increased percentage of these cells in G_1; (2) 'megaloblastoid' basophilic erythroblasts show a more pronounced decrease of their fraction in DNA synthesis than morphological normal cells; (3) 'megaloblastoid' early polychromatic normoblasts represent a non-proliferating cell population, and (4) the detected disturbance of cell proliferation – even though temporary – can be reversed.

Key Words
Autoradiography
Cytophotometry
DNA synthesis
Erythroleukaemia
Ineffective erythropoiesis
'Megaloblastoid' erythroblasts

Erythroleukaemia or DiGuglielmo syndrome is defined as an acute or chronic myeloproliferative disorder in which erythrocytes and their precursors are predominantly involved [3, 7]. Hyperplastic erythropoiesis is characterized by 'megaloblastoid' changes, nuclear abnormalities, giant erythroblasts and a strongly positive PAS reaction. Recent investigations give evidence that the erythropoiesis is highly ineffective [8]. Our work with the combined cytophotometric-autoradiographic method should help to answer the following questions: (1) Are the proliferating erythroblasts arrested in a defined phase of cell cycle? (2) Have 'megaloblastoid' erythroblasts other proliferative characteristics than the morphologically normal cells?

[1] We wish to thank Miss M. Brosius for excellent technical assistance.

Methods

Single cell suspensions of bone marrow were incubated for 1 h at room temperature with tritiated thymidine The concentration of ^{3}H thymidine was 2 μCi/ml, the specific activity 5 Ci/mmol Cell smears were fixed with absolute methanol (2×10 min) and stained by the May Grünwald Giemsa method Basophilic, early polychromatic and oxyphilic erythroblasts were photographed and the cell areas marked with an 'Objektmarker' (Leitz, Germany) on a second slide

Erythroblasts were classified according to KILLMANN [10] The May Grünwald Giemsa stain was then leached out with 5% trichloracetic acid (30 sec) and absolute methanol (10 min) and the smears were restained by the Feulgen method using a 9 min hydrolysis in 1 N HCl at 60 °C. The DNA content was measured with a cytophotometer UMSP I (Zeiss, Germany) at 560 nm The mean DNA value of at least 40 lymphocytes represented the diploid value

Autoradiographs were prepared by the dipping method at 18 °C, using the emulsion K5 (Ilford, England) Unstained smears were exposed at 2–4 °C for 7 days, smears stained by the Feulgen method for 21 days and developed 3 min with Kodak D19 at 18 °C Unlabelled cells with a diploid DNA content were in the presynthetic phase (G_1), all labelled cells were in the S phase Unlabelled cells with a tetraploid content of DNA could be found in G_2 or premitotic phase

Patients

The erythropoiesis of 5 patients (table I) 3 females and 2 males, was analyzed by the combined cytophotometric autoradiographic method In an additional case (No 1) only a cytophotometric examination was possible The erythropoiesis of patient 6 was examined 3 weeks after therapy with cytotoxic agents

All but one had a short anamnesis with anaemia and/or haemorrhagic diathesis Patient 2 suffered 51 months from an aplastic anaemia before erythroleukaemia appeared Initial severe anaemia was a common symptom (table I), thrombocytopenia was present in 5 of 6 patients In all instances erythroblasts were present in peripheral blood, their number varied between 40 and 17,500/mm^3 Bone marrow examination revealed a striking erythroid hyperplasia with 'megaloblastoid changes, multinucleated erythroblasts, and other nuclear abnormalities In 3 cases (No 1, 5, 6) a typical pattern of intense erythroblastic positivity of PAS reaction was observed The erythroblasts of the patients 1 and 5 showed excessive cytoplasmic vacuolization

Results

Erythroblasts were classified in 4 compartments E_1+E_2, E_3, E_4, and E_5 In each compartment 'megaloblastoid' and morphological normal cells were pooled Differential counts of erythropoiesis (table II) showed

Table 1 Main haematological data of the patients. The PAS reaction was evaluated according to the scoring method of QUAGLINO and HAYHOE [11]

Patient No.	Age	Hb g/100 ml	WBC $\times 10^3/\mu l$	Platelets $\times 10^3/\mu l$	Erythroblasts/μl	Erythropoiesis: erythropoiesis, %	Erythropoiesis: cytoplasmic vacuoles	Erythropoiesis: PAS reaction	Erythropoiesis: Giant cells	Erythropoiesis: Megaloblasts'	Erythropoiesis: Nuclear abnormalities
1	73	7.9	2.0	32.0	40	50	+	202	+	+	+
2	39	9.3	6.0	22.0	1 100	69	0	30	+	+	+
3	28	7.3	2.8	90.0	350	90	0	–	+	+	+
4	18	6.9	3.7	150.0	110	64	0	35	+	+	+
5	38	6.9	3.9	11.0	110	52	+	152	+	+	+
6	62	6.6	21.0	21.0	17,500	95	0	55	+	+	+

Table II Erythroblasts in bone marrow differential count, in each case 500 erythroblasts were differentiated

Patient No	E_1+E_2	E_3	E_4	E_5
1	1	0 7	0 7	0 4
2	1	1	1 6	1 6
3	1	0 9	1 7	1 3
4	1	0 6	1	1 1
5	1	0 4	0 4	0 7
6	1	0 6	1	1 1
Normal values (6 cases)	1	1 3	3 0	7 4

an increased percentage of immature forms E_1 and E_2, a disturbed pro portion of E_1+E_2 E_3 E_4 E_5 of 1 0 7 1 1 1 1 (mean values) was obtained

Table III contains the results of the combined cytophotometric-autoradiographic study In 4 cases (No 2–5) a markedly increased percentage of basophilic erythroblasts (E_1–E_3) and early polychromatic normoblasts (E_4) in G_1 with a decrease of DNA-synthetizing cells was found, quantitative differences between these 4 cases are evident Only in early polychromatic normoblasts a decreased S G_2 ratio was obtained, *the percentage of cells in premitotic phase was high in comparison to that* in DNA synthesis

In 2 cases (No 2, 5) the distribution of 'megaloblastoid' and morphol ogical normal erythroblasts in the various stages of the cell cycle were compared (table IV) It is obvious that in both cases 'megaloblastoid basophilic erythroblasts either show a more pronounced decrease of their portion in S or an increase in G_1 than morphological normal cells (fig 1) This change can be recognized in both procrythroblasts, macroblasts (E_1, E_2) and basophilic normoblasts (E_3) (table V) 'Megaloblastoid' E_4 of patient 3 are almost solely in G_1, normal cells showed still an increased percentage in G_1 (fig 2) The early polychromatic normoblasts of patient 2 showed marked 'megaloblastoid' changes, normal cells could not be identified, these cells are a non proliferating cell population (table III) Another case (No 6) only shows a lower S G_2 ratio in the compartment E_4 The erythropoiesis of this patient was examined 3 weeks after therapy with cytotoxic agents

Finally, in case 1 only a cytophotometric investigation of erythropoies-is was obtainable Most of the early polychromatic normoblasts had DNA

Table III Distribution (%) of basophilic erythroblasts (E_1-E_3) and early polychromatic normoblasts (E_4) in the various stages of cell cycle

Patient No	E_1-E_3						E_4					
	n	G_1	S	G_2	U	S G_2	n	G_1	S	G_2	U	S G_2
1	243						–	–	–	–	–	–
2	287	31.0	41.5	4.0	1.5	10.5	204	92.0	2.5	2.5	2.0	1.0
3	364	37.2	39.0	3.3	0.5	11.8	299	79.0	16.7	2.7	1.6	6.0
4	194	33.0	60.0	3.3	1.5	17.0	152	62.0	29.0	7.5	1.5	4.0
5	291	49.0	56.3	4.5	0	12.5	107	57.4	34.5	7.4	0.9	4.6
6	218	20.0	77.5	2.0	0.5	35.0	220	24.2	55.5	16.5	1.3	3.7
Normal values (5 cases)	898						917					
Mean		16.2	73.3	6.6		11.3		33.0	55.5	5.5		10.0
Range		11-22.6	73.5-77.0	3.0-13.5	<0.9	5.5-24.5		29-47.5	46.5-66.5	5.0-6.2	<0.9	7.8-10.7

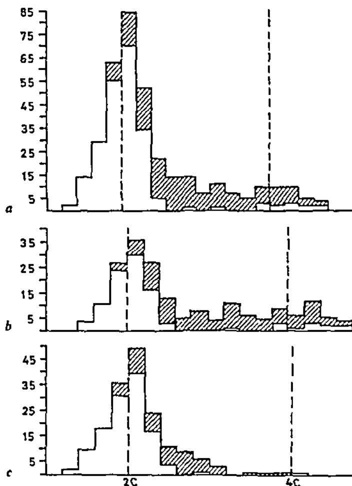


Fig 1 Patient 3 Relative DNA content and ^{3}H TdR labelling (shaded areas) of basophilic erythroblasts White areas = cells not labelled with ^{3}H TdR, *a* All cells *b* Morphologically normal cells *c* Megaloblastoid cells Ordinate = Number of cells, abscissa = DNA content (2c = diploid, 4c = tetraploid)

values near diploid quantity, while basophilic erythroblasts attained hexaploid values (fig 3) Multinucleated cells are not included in this figure

Discussion

In 1960 GAVOSTO *et al* [4] reported already that erythroblasts in erythroleukaemia have a lower labelling index than in healthy individuals Investigations by *in vivo* labelling with tritiated thymidine confirmed

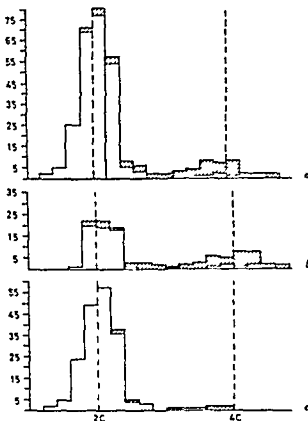


Fig. 2 Patient 3. Relative DNA content and ^{3}H TdR labelling (shaded areas) of early polychromatic normoblasts. White areas = cells not labelled with ^{3}H TdR. *a* All cells. *b* Morphologically normal cells. *c* Megaloblastoid cells. Ordinate = Number of cells; abscissa = DNA content (2c = diploid; 4c = tetraploid).

these results and revealed a highly ineffective erythropoiesis in erythroleukaemia [8]. Combined cytophotometric autoradiographic investigations on erythropoiesis so far resulted in contradictory findings. WICKRAMASINGHE *et al.* [3] in one case of erythroleukaemia demonstrated an arrest of early polychromatic normoblasts in the premitotic phase, while in a second case QUERINJEAN *et al.* [12] detected an increased percentage of proliferating erythroblasts in G_1. Finally CROSSEN *et al.* [2] in a third case found an arrest of cells in the S phase.

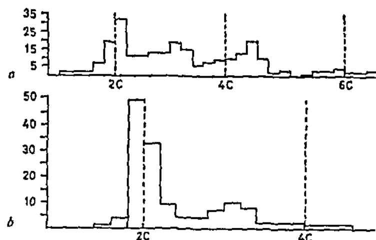


Fig 3 Patient 1 Relative DNA content of basophilic erythroblasts (a) and early polychromatic normoblasts (b) Ordinate=Number of cells, abscissa=DNA content (2c=diploid, 4c=tetraploid)

Table IV Distribution (%) of normal and 'megaloblastoid erythroblasts in the various stages of cell cycle In case 2 all early polychromatic normoblasts were 'megaloblastoid'

Patient No		E_1-E_3				E_4			
		G	S	G_2	U	G_1	S	G_2	U
2	all cells	53 0	41 5	4 0	1 5	–	–	–	–
	normal cells	48 0	46 5	4 5	1 0	–	–	–	–
	'megaloblastoid' cells	84 0	14 0	0 0	2 0	92 0	2 5	2 5	2 0
3	all cells	57 2	39 0	3 3	0 5	79 0	16 7	2 7	1 6
	normal cells	46 0	48 5	5 0	0 5	54 0	38 7	7 3	0 0
	'megaloblastoid' cells	70 6	28 0	0 7	0 7	93 1	4 2	0 0	2 7

Table V Patient 3 Distribution (%) of normal and 'megaloblastoid' proerythroblasts and macroblasts (E_1+E_2) and basophilic normoblasts (E_3) in the various stages of interphase

	E_1+E_2			E_3		
	G_1	S	G_2	G_1	S	G_2
All cells	51 5	44 5	4 0	64 5	33 0	2 0
Normal cells	38 5	55 0	6 5	53 0	42 0	5 0
'Megaloblastoid' cells	64 8	34 0	1 2	72 0	28 0	0

Our own results show (1) that the percentage of DNA-synthetizing basophilic erythroblasts and especially early polychromatic normoblasts is decreased while the number of cells in G_1 is increased, (2) that 'megaloblastoid' basophilic erythroblasts are more infrequent in DNA synthesis than morphologically normal cells, (3) that 'megaloblastoid' early polychromatic normoblasts are a non proliferating cell population. With regard to the results obtained by the *in vivo* labelling of erythropoiesis in erythroleukaemia [8] the disturbance of erythropoietic cell proliferation can be described as follows. A fraction of the proliferating erythroblasts is arrested in G_1; this may occur already in the compartment E_1 and E_2. In more mature compartments the percentage of cells arrested in G_1 increase, these cells develop a 'megaloblastoid' appearance. The 'megaloblastoid' early polychromatic normoblasts can amount to 100% of the total cell population (table IV) and do not proliferate (G_0 cells). They may die intramedullary or differentiate without mitosis to late polychromatic normoblasts. The disturbed cell proliferation leads to the hyperplasia and a shift to the left (table II) of erythropoiesis. The morphologically normal erythropoiesis, however, has a reduced proliferative activity and is comparable with erythropoiesis in refractory anaemia with hyperplastic bone marrow (unpublished results) and with erythropoiesis in acute leukaemia [9, 12].

Our results in case 6 (table III) show that the disturbance of cell proliferation – even though temporary – can be suspended. The erythropoiesis was studied 3 weeks after therapy with cytostatic agents; at this time the erythropoiesis was in a regenerating stage following cytostatically induced bone marrow aplasia.

The cytophotometric DNA measurements in case 1 show that a portion of mononucleated basophilic erythroblasts attained hexaploid values. Similar results have been obtained in investigations of blast cells in acute leukaemia [5] and are explained by a strong aneuploidy. Cytogenetic studies indicate that chromosomal abnormalities are very similar in erythroleukaemia and acute leukaemia [1, 2, 6].

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Different Molecular Weight of the Plasmatic Erythropoietin Compared with the Renal and Splenic Erythropoietic Factors in the Rabbit

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Abstract. The authors have studied the molecular weight of the erythropoietic factors present in the blood, in the kidney and in the spleen of the rabbit, by means of ultrafiltration experiments performed by using selective permeability membranes. The erythropoietic factor present in the blood circulation shows a molecular weight of between 10000 and 30000. The factors detectable in the microsomal fraction of normal kidney, in the mitochondrial fraction of anaemic kidney and in the mitochondrial fraction of normal spleen show molecular weights of between 50000 and 100000. These data confirm the hypothesis already formulated regarding the renal origin of the splenic erythropoietic factor and the different nature of the renal erythropoietic factor compared with the plasmatic erythropoietin.

Key Words
Erythropoietin
Plasmatic erythropoietic factor
Renal erythropoietic factor
Selective ultrafiltration
Splenic erythropoietic factor

It is known that the kidney produces an erythropoietic factor that interacts with a plasmatic substratum to form erythropoietin [1, 11]. In the rabbit this factor accumulates at least partly in the spleen and is then released into the blood circulation, both in the first few hours after bleeding and in the subsequent evolution of the anaemia [6–8]. In addition, it has been shown that the erythropoietic factors present in the kidney and in the spleen of the rabbit reveal different chemicophysical characteristics from those of the plasmatic erythropoietin [2, 9].

In these studies we performed experiments using a series of selective permeability membranes capable of allowing the filtration of substances of gradually decreasing molecular weight. In this way we tried to obtain indications on the molecular weight of the various erythro-stimulant fac-

tors, in order to check the possible differences in weight between the renal and splenic factors on the one hand and the plasmatic factor on the other

These studies were suggested by the works of LEWIS *et al* [4, 5] who identified in the urine concentrates of anaemic patients, two erythro stimulant factors having biological activity of the erythropoietin type, one of them having a molecular weight of between 20 000 and 30 000 and the other of over 50 000 In the same material an erythropoietin generating factor (EGF) has also been revealed, having a molecular weight situated between 30 000 and 50 000, this factor is found to be capable of producing erythropoietin when incubated with serum of a normal rabbit

Material and Methods

The researches were carried out by studying the variations in the peripheral reticulocytes of normal rabbits by the administration of materials obtained by means of fractionation with ultrafiltration membranes from organ extracts or plasma extracts prepared from donor rabbits (6–8 months of age 3–3 5 kg in weight) some being normal rabbits and others having been bled by up to 20 ml/kg body weight

In particular on the basis of studies recently carried out on the subcellular localisation of the renal and splenic erythropoietic factors [3] the materials chosen as presumed source of these substances were hypotonic extracts obtained from the microsomal fraction of normal kidney the mitochondrial fraction of anaemic kidney (18 h after bleeding) and the mitochondrial fraction of normal spleen As presumed source of erythropoietin the materials used were plasma extracts from rabbits bled 24 h previously [2 9]

The hypotonic extracts of the subcellular fractions of renal and splenic origin were prepared according to the technique described in a previous work [3] On the other hand the plasma extracts were prepared by removing 50 ml anaemic plasma from each donor This material after the addition of 25 ml bidistilled water was acidified at pH 5 5 by means of 1 N HCl It was then heated in boiling water for 10 min in order to precipitate most of the proteins and was finally centrifuged The supernatant rich in erythropoietin was brought up to pH 7 with 1 N NaOH

The fractionation of the plasma or organ extracts was performed with an AMICON cell for ultrafiltration provided with a series of DIAFLO membranes having different cut off values of molecular weight With this device the materials studied were subjected to successive filtrations by means of membranes of increasing capacity of retention During filtration the cell was pressurised with nitrogen the pressures applied for the various membranes being those recommended by the makers

Each extract of anaemic plasma brought to a volume of 100 ml by the addition of bidistilled water was subjected to 2 successive ultrafiltrations by means of PM 30 and PM 10 membranes characterised by cut-off values of molecular weight equal to 30 000 and 10 000 respectively

Each hypotonic extract of the subcellular fractions used brought up to a volume

of 100 ml by the addition of bidistilled water, was subjected to 3 successive ultrafiltrations with XM 100-A, XM 50 and PM 10 membranes, having cut-off values of molecular weight equal to 100 000, 50 000 and 10 000, respectively.

Filtration through each membrane was performed twice, in order to obtain a more precise separation of the substances of molecular weight higher or lower than the cut-off values of the membrane used. In particular, the residual volume obtained after the first filtration, equal to about 5 ml, was brought up to 100 ml by the addition of bidistilled water before being subjected to a supplementary filtration. The various operations described were performed at a room temperature of 3 °C.

The residual material, obtained after each filtration was brought up to a volume of 40 ml by the addition of bidistilled water. The whole was made isotonic by means of NaCl and, after the addition of antibiotics, was kept at −20 °C until use. Every fraction diluted in this way was given to a receiver animal in 2 intraperitoneal injections of 20 ml each, administered 24 h apart.

The erythro-stimulant activity of each fraction was assessed by studying the reticulocyte counts in the receiver animals during the first 6 days. In every case the reticulocyte level was assessed on the basis of 2 000 erythrocytes, microscope samples being prepared with blood removed from the marginal vein of the ear and being stained with brilliant cresyl blue. Before the experiments, the rabbits (4–5 months of age, 2–2.5 kg in weight) were subjected on alternate days to 10 successive counts of the reticulocyte level and of the number of erythrocytes, so as to select for the experiments only those animals having an erythrocyte level of between 5,800 000 and 6,200 000 μl and a reticulocyte level of between 1.5 and 3%, and a standard error not greater than 0.1 in the 10 values. In this way it was possible to obtain highly stable and consistent experimental data.

In all, 4 series of experiments were performed, corresponding to the 4 materials studied (extracts of anaemic plasma and hypotonic extracts of the microsomal fraction of normal kidney, the mitochondrial fraction of anaemic kidney, and the mitochondrial fraction of normal spleen).

The first series of experiments was performed with the following groups of rabbits: (1) 6 donors bled 24 h previously, from which to remove the plasma for preparing the extracts to be subjected to fractionation with PM 30 and PM 10 membranes; (2) 12 receivers (i.e. 2 for each donor), to be given the fractions held back by the 2 types of membrane used in the experiment.

Each of the other 3 series of experiments was performed with the following groups of rabbits: (1) 6 donors, either normal or bled 14 h previously, from which to remove the kidneys or the spleen for preparing the extracts of the subcellular fractions considered. The extract of each fraction was subjected to ultrafiltration with XM 100, XM 50 and PM 10 membranes; (2) 18 receivers (i.e. 3 for each subcellular fraction used), to be given the subfractions held back by the 3 types of membrane used in the experiment.

Results

The results obtained from the treatment with extracts of anaemic plasma (table I) show that the fraction held back by the PM-30 membrane is

Table I Variations with time in the reticulocyte level in rabbits treated with fractions of plasma extract removed from donors bled 24 h previously, prepared by means of successive ultrafiltrations with DIAFLO membranes PM 30 (group I A) and PM 10 (group I B)

	Group I-A		Group I B	
	mean±SE	t test	mean±SE	t test
Initial	2 68±0 1558		2 65±0 1176	
2 days	2.7 ±0 1915	0 08103	3 78±0 147	6 003[1]
3 days	2 7 ±0 1807	0 08382	4 13±0 1306	8 419[1]
4 days	2 67±0 1819	0 04175	3 65±0 1432	5 397[1]
6 days	2 65±0 1911	0 1217	2 88±0 1815	1 064

The reticulocyte level is expressed as a reticulocyte/erythrocyte percentage Mean values±standard error and comparison between the averages of initial and subsequent values by means of Student s t test (degrees of freedom, 10)

[1] Highly significant

lacking in erythro-stimulant activity, whereas that held back by the PM-10 membrane shows such activity In fact, the specimens treated with the plasma fractions held back by the former membrane show no significant variation in the reticulocyte pattern (group I-A), whereas in the latter case (group I-B) they show a highly significant increase in the number of circulating reticulocytes

The experiments performed by administering the fractions obtained from the ultrafiltration of renal or splenic subcellular extracts led to substantially equal results, so that they have been assembled in a single table in order to facilitate comparative assessment (table II) It may be seen that in all the materials of renal and splenic origin used the erythropoietically active fraction is the one that passes through the XM-100-A membrane but is held back by the XM-50 membrane In fact, the treatment with the fractions held back by the XM-100-A membrane and obtained from the microsomal extract of normal kidney (group II-A), the mitochondrial extract of anaemic kidney (group III-A) and the mitochondrial extract of normal spleen (group IV-A) does not produce any significant variation in the reticulocyte number, whereas the treatment with the fractions relative to the same subcellular extracts, which pass through the XM-100-A membrane but are held back by the XM-50 membrane (groups II-B, III-B, IV-B), produces a very marked increase in the number of circulating reticulocytes In addition, the fractions capable of pass-

Table II. Variations with time in the reticulocyte level in rabbits treated with fractions obtained by successive ultrafiltrations with DIAFLO membranes XM 100-A (groups A), XM 50 (groups B) and PM 10 (groups C) from hypotonic microsomal extracts of kidney removed from normal donors (groups II), mitochondrial extracts of kidney removed from donors bled 18 h previously (groups III) and mitochondrial extracts of spleen removed from normal donors (groups IV)

	Group II A		Group II B		Group II-C	
	mean ± SE	t test	mean ± SE	t test	mean ± SE	t test
Initial	2.8 ± 0.1183		2.73 ± 0.1229		2.77 ± 0.1838	
2 days	2.83 ± 0.1406	0.1633	4.18 ± 0.1275	8.187[1]	2.7 ± 0.1897	0.265
3 days	2.85 ± 0.1558	0.409	4.53 ± 0.1256	10.24[1]	2.85 ± 0.2432	0.2624
4 days	2.9 ± 0.1592	0.5042	3.92 ± 0.1046	7.376[1]	2.83 ± 0.212	0.3921
6 days	2.85 ± 0.1869	0.3617	2.97 ± 0.1305	1.337	2.95 ± 0.1928	0.6759

	Group III A		Group III B		Group III-C	
	mean ± SL	t test	mean ± SL	t test	mean ± SL	t test
Initial	2.93 ± 0.0943		2.45 ± 0.1409		2.73 ± 0.1201	
2 days	3 ± 0.1183	0.4819	3.83 ± 0.2170	5.334[1]	2.93 ± 0.1382	1.092
3 days	2.95 ± 0.0972	0.4124	4.15 ± 0.2277	6.35[1]	2.93 ± 0.1145	1.205
4 days	3.2 ± 0.1265	1.776	3.6 ± 0.1732	5.152[1]	3.02 ± 0.1137	1.753
6 days	3.22 ± 0.1275	1.897	2.9 ± 0.1653	2.072[1]	2.95 ± 0.0935	1.474

	Group IV-A		Group IV B		Group IV-C	
	mean ± SL	t test	mean ± SL	t test	mean ± SL	t test
Initial	2.8 ± 0.1844		2.8 ± 0.1342		2.65 ± 0.1661	
2 days	2.85 ± 0.1780	0.3156	4.25 ± 0.1945	6.137[1]	2.8 ± 0.1953	0.4638
3 days	2.85 ± 0.1335	0.2196	4.53 ± 0.2216	6.679[1]	2.77 ± 0.1429	0.4107
4 days	2.95 ± 0.1962	0.5571	3.9 ± 0.1483	5.5[1]	2.72 ± 0.1400	0.1841
6 days	2.93 ± 0.1960	0.433	2.97 ± 0.1646	0.8005	2.85 ± 0.1922	0.7545

The reticulocyte level is expressed as a reticulocyte erythrocyte percentage. Mean values ± standard error and comparison between the averages of the initial and subsequent values by means of Student's t test (degrees of freedom, 10).
[1] Highly significant.

ing through the XM 50 membrane but held back by the PM-10 membrane do not reveal any significant erythro-stimulant activity (groups II-C, III-C, IV-C). The increase in the number of circulating reticulocytes was always accompanied by an increase in the number of erythrocytes

Discussion

From the results obtained in the first series of experiments it may be seen that the molecular weight of the erythropoietin present in the plasma of anaemic rabbits is between 10,000 and 30,000 This observation would appear to agree with the value of 27,000 proposed by Rosse *et al* [10] and with the results of the more recent studies carried out by Lewis *et al* [5], who found, in the urine of anaemic patients, an erythropoietin having a molecular weight of between 20,000 and 30,000

The results obtained in the other experiments show that the erythro stimulant factors previously identified in the microsomal fraction of normal kidney, the mitochondrial fraction of anaemic kidney and the mitochondrial fraction of normal spleen [3] have a molecular weight of between 50,000 and 100,000

First of all, the analogies existing between the erythropoietic factors present in the 2 different renal fractions studied is in complete agreement with the already advanced theory [3] of an accumulation of the renal factor considered in the mitochondrial fraction, when, as a result of anaemia, its production increases considerably Moreover, the substantial analogy in the behaviour of the renal and splenic erythro-stimulant factors, in relation to their ability to pass through the membranes, may be considered a further confirmation of the theory previously proposed regarding their identical nature [2, 7–9]

The results obtained in the present studies also confirm that these renal and splenic factors cannot be absolutely identified with plasmatic erythropoietin, as was often proved in previous studies [2, 9] In fact, erythropoietin forms through interaction between a factor released into the blood circulation by the kidney or by the spleen and a plasmatic substratum

It is possible that the erythro stimulant factors identified in the kidney and in the spleen [2, 3, 8, 9] may have an origin similar to that of the EGF identified by Lewis *et al* [4, 5] in the urine of anaemic patients, even if this EGF shows a lower molecular weight of between 30,000 and 50,000 It is possible that this factor, once its cycle of activity in the blood circulation has been exhausted and it has been excreted by the kidneys, may have undergone structural modifications such as to produce a reduction in its molecular weight, though without change of its biological activity

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Acta haemat 53 82-89 (1975)

Desaggregation of Human Red Blood Cells by Various Surface-Active Agents as Related to Changes of Cell Shape and Hemolysis

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Abstract The effect of several surface active substances (Na oleate, Na desoxycholate Triton X 100 Pluronic F 38 Pluronic F 68 Pluronic F 108) on dextran induced red cell aggregation (RCA) and red cell shape was investigated *Sedimentation measurements and photometric determinations* of RCA indicated a desaggregating effect of oleate, Triton, F 38 and F 68 Shape changes were found in the presence of oleate and desoxycholate (crenation) and Triton (cup formation), in addition, these substances induced pronounced hemolysis No hemolysis and no shape changes were found with any of the Pluronic polyols

Key Words
Aggregation of erythrocytes
Erythrocyte desaggregation
Erythrocyte sedimentation
Erythrocyte shape
Hemolysis
Surface-active substances

It is generally accepted that increased aggregation of red blood cells (RBC) is brought about by high molecular compounds, and the hypothesis has been forwarded that this effect is due to the formation of molecular bridges between adjacent membranes of neighboring RBC [1] It is also known that deviations from the normal biconcave RBC shape will eventually result in desaggregation, this may be due to a reduction of the area of contact available In addition, surface tension effects may be involved since many of the compounds which lead to shape changes also show surface active properties In the present study, the desaggregating action of various surface-active substances is investigated and related to changes of cell shape and hemolysis

Materials and Methods

Blood was obtained from healthy human donors by venipuncture and anticoagulated with heparin (5 IU/ml) 4 0 ml blood were incubated with 0 5 ml of a 2% solution of dextran (MW 510 000) (supplied by Knoll AG Ludwigshafen Germany) in saline Equi

molar solutions (20, 10, 5, 2 mM) of the substances to be tested were prepared in saline and 0.5 ml of these solutions were added to 4.5 ml HMD blood while the control samples received 0.5 ml saline. Thus, the final concentrations of the substances tested were 2, 1, 0.5, 0.2, and 0 mM.

Since equal volumes of the solutions were added to equal volumes of blood, all comparisons were made at a constant plasma dilution by approximately 2°/₀, depending upon hematocrit. The following compounds were investigated: Pluronic® F 38, Pluronic® F 68 (Lutrol, supplied by C. H. Erbslöh, Düsseldorf, Germany; BASF Wyandotte Corporation, Wyandotte, Mich., USA), Triton X 100 (Rohm & Haas, Philadelphia, USA), Pluronic® F 108 (supplied by BASF Wyandotte Corporation, Wyandotte, Mich., USA), Na-oleate (Riedel-de Haan, Seelze-Hannover, Germany), and Na-desoxycholate (E. Merck, Darmstadt, Germany).

Red cell aggregation was determined indirectly by measuring sedimentation velocities in standard Westergren tubes at room temperature. The height of the cell plasma interface was read every 10 min and the maximum slope of the plot of interface versus time was determined. These data were corrected for changes of suspending medium viscosity which was measured in an Ostwald capillary viscometer at room temperature. The corrections were made by taking the suspending medium viscosity of the control sample (containing 0.5 ml HMD solution and 0.5 ml saline) as 100°/₀.

Additional determinations of red cell aggregation were made photometrically. A sample of blood was drawn at a rate of 24.5 ml/min through an oxymetry cuvette (Atlas oxymeter) which was mounted horizontally such that the light passed vertically throught the layer of blood. The transmission of light was recorded continuously on a paper recorder. After stopping the flow of blood through the cuvette, the light transmission increased due to formation of red cell aggregates. The extent of aggregation was determined by measuring the increase of light transmission observed during a period of 1 min.

Microphotographs of the different cell suspensions were made by preparing wet mounts and taking pictures 1 min after the preparation using a Leitz Ortholux microscope. A magnification of 450 × was used, employing 10 × Periplan eyepieces and 45 × Leitz objectives.

Using the cyanmethemoglobin technique, hemoglobin concentrations were measured in the supernatant suspending medium after centrifugation of the samples.

Results

The effect of the different substances tested on RBC sedimentation velocity is shown in table I. A reduction of the sedimentation velocity was observed in the presence of Pluronic F 38, F 68, Triton X 100, and Na-oleate. Whereas with the first 3 substances the sedimentation velocity was reduced in an approximately linear fashion with increasing concentration, this was different in the case of Na-oleate which showed little effect up to approximately 1 mM/l but was very effective at 2 mM/l (p < 0.01). At 2 mM/l Pluronic F 38, Triton X 100, and Na-oleate were equally effective, whereas the effect particularly of F 68 was relatively weak. In contrast to these ob-

Table I Maximum sedimentation velocities (V_{max}) of human red blood cells in samples containing surface active substances, all values were corrected for changes in suspending medium viscosity (η_s), mean values and standard deviations

Concentration mM/l	Pluronic F 38		Pluronic F 68		Pluronic F 108		Triton X 100		Na-desoxycholate		Na-oleate	
	V_{max}	η_s	V_{max}	η_s	V_{max}	η_s	V_{max}	η_s	V_{max}	η_s	V_{max}	η_s
0	12.08	1.619	10.89	1.619	10.17	1.619	12.08	1.619	12.08	1.619	12.08	1.619
	±2.73	±0.141	±3.03	±0.141	±2.50	±0.141	±2.73	±0.141	±2.73	±0.141	±2.73	±0.141
0.2	11.14	1.606	8.88	1.675	11.59	1.828	11.59	1.626	11.44	1.581	12.06	1.581
	±3.08	±0.130	±2.89	±0.104	±3.21	±0.047	±3.48	±0.134	±3.87	±0.151	±3.73	±0.124
0.5	11.18	1.671	7.53	1.818	12.13	2.170	7.75	1.621	11.33	1.575	10.92	1.584
	±3.27	±0.155	±2.99	±0.145	±3.66	±0.190	±3.12	±0.135	±4.69	±0.139	±3.59	±0.137
1.0	9.35	1.752	5.07	2.009	6.13	2.596	6.62	1.649	12.81	1.579	11.36	1.583
	±3.28	±0.157	±2.60	±0.168	±2.61	±0.401	±3.75	±0.148	±6.07	±0.172	±2.84	±0.136
2.0	6.99	1.915	1.06	2.406	28.59	4.099	1.92	1.816	19.17	1.681	1.01	1.615
	±1.97	±0.177	±0.53	±0.236	±21.68	±0.552	±1.00	±0.210	±11.23	±0.135	±1.03	±0.139

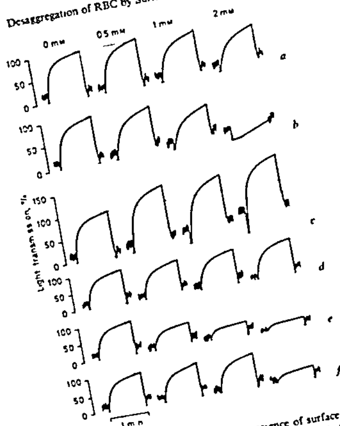


Fig. 1. Photometric measurements of RCA in the presence of surface active agents. *a* = Pluronic F 38, *b* = Pluronic F 68, *c* = Pluronic F 108, *d* = Na-desoxycholate, *e* = Triton X 100, *f* = Na-oleate

servations statistically significant effects were not observed in the case of Na-desoxycholate. With F 108 a biphasic effect was obtained: a slight decrease of sedimentation velocity (p 0.025) at 1 mM/l and an increase of sedimentation velocity at 2 mM/l (p 0.05).

Table I also gives the changes of suspending medium viscosity observed. The largest effect was measured in the samples containing F 108; here, the suspending medium viscosity increased from an average of 1.619 ± 0.141 in the control samples to 4.099 ± 0.552 at a concentration of 2 mM/l. This increase was somewhat less in the case of F 68, where an average of 2.506 ± 0.236 was found at the highest concentration, and in the case of F 38, where

Table II Hemoglobin concentrations (g%) in the suspending medium after incubation of human blood with increasing concentrations of surface-active substances, mean values and standard deviations of 6 experiments

Concentration mM/l	Triton X 100	Na-desoxycholate	Na-oleate
0	0 177±0 092	0 177±0 092	0 177±0 092
0 2	0 299±0 168	0 181±0 078	0 201±0 089
0 5	0 386±0 104	0 207±0 141	0 155±0 099
1 0	0 652±0 234	0 379±0 233	0 198±0 073
2 0	2 062±0 748	1 043±0 272	0 363±0 097

at 2 mM/l the average viscosity was 1 915±0 177 Only a minor increase was measured in the presence of Triton X 100, whereas no statistically significant effect was found with Na oleate and Na desoxycholate

The results of the photometric measurements are represented in figure 1 After stopping the blood flow through the cuvette, the light transmission decreased immediately due to randomization of cell orientation under conditions of stasis Very rapidly, however, the process of RBC aggregation resulted in an increase of light transmission The change of photovoltage ΔV occurring in a period of 1 min in the sample containing only 2% HMD was taken as 100% The changes of ΔV in the presence of the 6 surface-active substances were corrected for changes in suspending medium viscosity η_s by multiplying ΔV with the ratio of η_s and the viscosity of the control medium η_s The corrected increase of photovoltage $\Delta V = \Delta V \cdot \eta_s/\eta_c$ in the presence of the highest concentrations of the compounds tested was as follows Pluronic F 38 106 2%, Pluronic F 68 55 1%, Pluronic F 108 402 3%, Triton X 100 29 9%, Na oleate 36 1%, Na desoxycholate 112 7%

Figure 2 shows photomicrographs of blood samples containing the 6 compounds tested in a concentration of 2 mM/l No dextran was added to these samples In the presence of Na oleate or Na desoxycholate, the red blood cells were crenated, whereas in the presence of Triton X 100 they had adopted a cup like shape In the samples containing the 3 Pluronics no significant shape changes were observed Intensified red cell aggregation was found in the samples containing F 108, whereas in the presence of F 68 virtually no aggregates were observed

The results of the hemoglobin determinations in the suspending medium are shown in table II Significant hemolysis was observed only in the samples

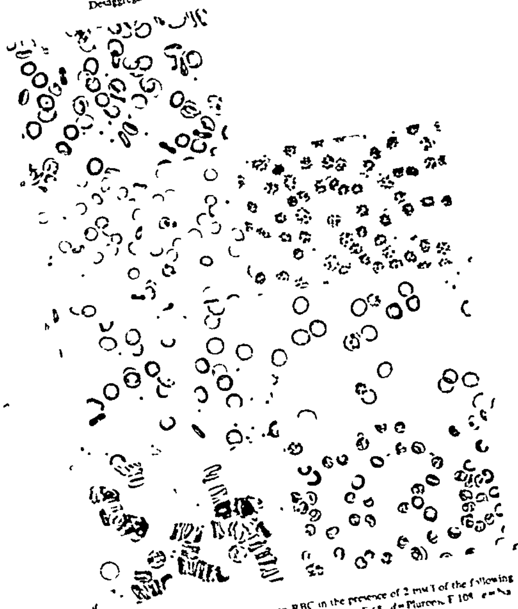


Fig. [illegible] Photomicrographs of human RBC in the presence of 2 mM of the following substances: *a* = Control, *b* = Pluronic F 38, *c* = Pluronic F 68, *d* = Pluronic F 108, *e* = Na oleate, *f* = Na-deoxycholate, *g* = Triton X 100

containing Triton X 100, Na-desoxycholate, and Na-oleate. In these samples, hemoglobin levels increased with increasing concentrations of these substances. Triton X 100 was found to have the strongest hemolytic activity, followed by Na-desoxycholate and Na-oleate. At the highest concentration of these substances (2 mM/l), the hemoglobin concentrations in the suspending medium averaged 2.062 ± 0.748 g% in the presence of Triton X 100, 1.043 ± 0.272 g% in the presence of Na-desoxycholate, and 0.363 ± 0.097 g% in the presence of Na-oleate. In contrast to these results, no hemolysis was observed in the samples containing any of the Pluronic compounds.

Discussion

The results of the experiments indicate that in the presence of Triton X 100, Na-oleate, Pluronic F 38 and Pluronic F 68 the RBC aggregation induced by high molecular dextran is inhibited in a dose-dependent fashion. This is obvious from the sedimentation velocity data, which after correction for suspending medium viscosity reflect changes of average particle size, corrections for changes of particle and/or suspending medium density [5] have, however, not been made. The results of the photometric measurements support the conclusions drawn from the sedimentation experiments by again demonstrating a desaggregating effect of the above-mentioned compounds. In these measurements, corrections for changes of density are not necessary since the results are not influenced by sedimentation in the measuring cuvette.

The desaggregation occurring in the presence of Na-oleate and Triton X 100 can be attributed to the shape changes observed microscopically, which have also been observed by other authors [2, 3]. The decreased tendency to aggregation of RBC which do not show the normal biconcave shape can be attributed to a reduction of contact area between adjacent cells. This has already been hypothesized [6] in explaining the decrease of yield shear stress observed in hypertonic media.

Obviously, desaggregation can be the result of either crenation (Na-oleate) or cup formation (Triton X 100). Both substances also are found to produce hemolysis which in our experiments was approximately 10 times stronger in the case of the cup-producing compound in comparison to the crenating compound. Na-desoxycholate, on the other hand, showed insignificant effects on RBC aggregation, this may be compared to its lesser crenating action. In contrast, the hemolysis found in the presence of Na-

desoxycholate was significantly stronger in comparison to that of Na oleate. It appears therefore that desaggregation of human RBC is more closely related to changes of cell shape than is hemolysis.

No shape changes and also no hemolysis was found in the presence of either of the 3 Pluronic polyols. In contrast, previous studies in this laboratory [4] have shown an antihemolytic action of these compounds, notably of F 68. Consequently, the desaggregating effect of F 68 must be attributed to a mechanism different from that underlying the effect of the low molecular compounds used in this study. Whereas interaction with charged groups on the RBC surface or within the cell membrane might provide the basis for shape changes in the presence of anionic or cationic substances [2], additional mechanisms must be hypothesized to explain the action of the Pluronic polyols. It appears conceivable that adsorption of these compounds onto the cell membrane might interfere with the intercellular bridging effect brought about by the dextran molecules [1].

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Acta haemat 53 90-97 (1975)

Vitamin B_{12} and Vegetarianism in India

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Abstract Apparently healthy Indian lactovegetarians have significantly lower serum levels and urinary excretion of vitamin B_{12} as compared to non vegetarians The low levels of serum vitamin B_{12} in lactovegetarians are not due to defective absorption but to low dietary intake of this vitamin Inspite of considerably low serum vitamin B_{12} values the lactovegetarians have no apparent signs or symptoms of vitamin B_{12} deficiency and their response to injected cyanocobalamin mixed with tracer radioactive B_{12} as judged by urinary excretion pattern and liver uptake, was similar to that observed in non vegetarians

Key Words
Lactovegetarians
Liver B_{12} uptake
Serum B_{12}
Urinary B_{12} excretion
Vegetarianism
Vitamin B_{12}

In India, a majority of the people consume a vegetarian diet from childhood with occasional supplement of milk or dairy products, because of religious convictions and high cost, intake of non-vegetarian food is relatively little Purely on the basis of serum vitamin B_{12} levels, large part of the Indian population could be considered deficient in this vitamin [2, 7, 15, 18] However, the incidence of megaloblastic anaemia or neurological disease due to B_{12} deficiency in India does not appear to be high [5] In fact, the lactovegetarian volunteer students with low serum vitamin B_{12} levels showed no clinical evidence of vitamin B_{12} deficiency [15, 18] The present study was undertaken to investigate further the signifi-

cance of serum vitamin B_{12} levels in apparently healthy Indian subjects with various dietetic habits

Material and Methods

The subjects included in this study consisted of medical students between the ages of 18 and 26 years. The detailed history was obtained as regards their height, weight, previous illness and dietary habits with particular reference to the type of protein in their diet. None of these had taken any vitamin supplements at least for 4 months prior to this study. The basic food in the whole group consisted of varying amounts of polished rice, wheat flour in the form of chapaties, legumes, cooked green vegetables and occasional fruits. The vegetarians who were taking milk were grouped as lactovegetarians; their main source of protein was of vegetable origin in addition to 100–250 ml milk/day. Subjects who were consuming non-vegetarian food like meat, fish, or fowl as often as once a day were grouped as non-vegetarian frequent meat eaters, while those who took non-vegetarian food irregularly were classified as *non-vegetarian occasional meat eaters*.

Venous blood samples were collected in the morning before breakfast. Subjects were, however, allowed to take a cup of tea or coffee. Blood was allowed to clot at room temperature and the serum was separated by centrifugation.

Serum vitamin B_{12} was determined by the method of ROSS [17] using *Euglena gracilis* var. *bacillaris* as the test organism. All estimations were done in duplicate.

Total plasma protein was determined by specific gravity method using copper sulphate standards. Haemoglobin, packed cell volume, red cell count and neutrophil lobe count were measured and MCV, MCH and MCHC were calculated by standard methods [21].

Urinary excretion and hepatic uptake of vitamin B_{12} following the administration of cyanocobalamin was studied using ^{58}Co-labeled cyanocobalamin obtained from Bhabha Atomic Research Centre, India. For this purpose, a tracer dose of 1 µCi of ^{58}Co-labeled cyanocobalamin mixed with 500 µg of cyanocobalamin was injected intramuscularly. Seven lactovegetarians and five non-vegetarian, frequent meat eaters volunteered for this study. The subjects were allowed to take routine diet. Injections were made in the deltoid region and the radioactivity injected was measured immediately by using scintillation probe. 24 Hour urine samples were collected during a period of 3 days and radioactivity excreted in terms of percent of the dose given was calculated.

The hepatic uptake of ^{58}Co-labeled cyanocobalamin was determined by the surface-counting technique described by GLASS *et al.* [10] using a scintillation detector connected to a rate meter. Three cutaneous projections, namely anterior, anterolateral and lateral of the liver were counted for calculating the mean and necessary corrections for background and physical decay were made. As it takes about 4–6 days to build up the peak of radioactivity over the liver, the hepatic measurements were carried out on 3rd, 5th and 7th day after the injection.

Intestinal absorption studies with radioactive vitamin B_{12} were carried out as described by SCHILLING [20].

Table I Haematological data and mean serum vitamin B_{12} levels in Indian subjects (Mean values with ± SE)

Group	Number of cases	Hb g/100 ml	PCV %	RBC $\times 10^6/\mu l$	Neutrophil lobe count	MCV μm^3	MCH pg	MCHC %	Vitamin B_{12} pg/ml
Males									
Lactovegetarians	78	15.5 ± 0.12	46 ± 0.49	5.21 ± 0.07	3.51 ± 0.04	88 ± 1.29	29 ± 0.42	33 ± 0.24	107.4 ± 9.37
Non vegetarian, occasional meat eaters	85	15.4 ± 0.12	46 ± 0.50	5.32 ± 0.07	3.50 ± 0.04	87 ± 1.25	29 ± 0.38	33 ± 0.22	205.0 ± 11.49
Non vegetarian, frequent meat eaters	34	15.6 ± 0.13	47 ± 0.42	5.41 ± 0.11	3.43 ± 0.03	87 ± 1.14	29 ± 0.36	33 ± 0.20	379.3 ± 28.40
Females									
Lactovegetarians	38	13.6 + 0.25	40 ± 0.66	4.51 ± 0.07	3.47 ± 0.07	88 ± 1.80	30 ± 0.65	34 ± 0.32	136.4 ± 11.87
Non vegetarian, occasional meat eaters	15	13.2 ± 0.23	40 ± 0.71	4.91 ± 0.17	3.45 ± 0.05	83 ± 3.04	27 ± 1.13	33 ± 0.53	202.3 ± 20.91
Non vegetarian, frequent meat eaters	12	13.6 ± 0.15	40 ± 0.60	4.69 ± 0.11	3.53 ± 0.07	86 ± 2.00	29 ± 0.69	34 ± 0.48	390.9 ± 64.95

Results

Haematological Data, Serum Vitamin B_{12} and Diet

Serum vitamin B_{12} levels in 197 males and 65 females, analysed according to their diet are given in table I The groups were similar in respect to age, height, and weight The mean haemoglobin packed cell volume, red blood cell count, MCV, MCH, MCHC and neutrophil lobe count values in lactovegetarians were not significantly different from those observed in frequent non vegetarians However, the mean serum vitamin B_{12} level among the lactovegetarians was nearly one third of the mean level observed in non vegetarians Further, 42 lactovegetarian males and 13 lactovegetarian females had serum vitamin B_{12} values less than 100 pg ml and in 19 of these, the level was as low as 50 pg/ml None of these subjects showed any clinical or haematological evidence of vitamin B_{12} deficiency Non vegetarian occasional meat eaters had serum vitamin B_{12} values in between these two groups Serum vitamin B_{12} levels did not differ significantly between male and female subjects although the mean weight, haemoglobin, packed cell volume and red blood cell count in females were lower than those observed in male subjects Non-vegetarian frequent meat eaters had serum vitmain B_{12} levels comparable to those reported by Western workers [4]

The mean 24 hour urinary excretion of vitamin B_{12} was 36 54 ng (range 9 3–80 3 ng) in 10 lactovegetarians as compared to 69 11 ng (range 18 5–147 0 ng) in 18 frequent non vegetarian subjects

Urinary Excretion and Hepatic Uptake following Cyanocobalamin Injection

The measurement of the urinary radioactivity following intramuscular injection of 500 μg of cyanocobalamin mixed with ^{58}Co-labeled cyanocobalamin revealed the excretion of the major portion of the injected dose within first 24 h It was 69 8 + SE 2.39% of the dose in lactovegetarians and 64 8 + SE 4 09% in non vegetarian, frequent meat eaters The statistical comparison showed no significant difference in the total 24 hour radioactivity excreted in these two groups During subsequent 48 h total 1 79 + SE 1 33% of the radioactivity was excreted by the lactovegetarians and 3 14 + SE 0 69% by the non vegetarian frequent meat eaters

Studies on the hepatic uptake of the radioactive cyanocobalamin showed the mean hepatic uptake of 1 74 ± SE 0 23% of the injected dose on 3rd day 1 87 + SE 0 22% on 5th day and 1 94 ± SE 0 25% on 7th

day in lactovegetarians Corresponding figures in non-vegetarian, frequent meat eaters were 1 95 ± SE 0 27%, 1 97 ± SE 0 09%, and 2 08 ± SE 0 15%, respectively Statistical analysis revealed no significant difference

Intestinal Absorption of Radio-Vitamin B_{12}

In order to study intestinal absorption of vitamin B_{12}, Schilling test was carried out in 10 lactovegetarians and 10 non-vegetarian frequent meat eaters of comparable age, weight, and height ranges The mean radioactivity excretion following the flushing dose of 1,000 μg of stable cyanocobalamin was 23 4 ± SE 2 66% in lactovegetarians while it was 11 6 ± SE 1 82% in non-vegetarian, frequent meat eaters The difference is statistically significant ($p < 0\ 01$)

Discussion

These results confirm previous findings that the healthy Indian lactovegetarians have markedly lower serum vitamin B_{12} levels than those in non-vegetarians [15, 18] The lactovegetarians did not present any abnormal clinical findings and their blood picture, as judged by haemoglobin, packed cell volume, neutrophil lobe count, plasma proteins and various blood indices, did not significantly differ from that observed in the non-vegetarian group The low serum vitamin B_{12} levels observed in lactovegetarians could be either due to deficiency of this vitamin in the diet or its defective intestinal absorption The results of Schilling test showed normal absorption of this vitamin as compared to non-vegetarians The low serum vitamin B_{12} levels in lactovegetarians were, therefore, due to low dietary intake of this vitamin Since these subjects were habitual vegetarians such levels must be present for prolonged time

Although purely on the basis of dietary analysis, clinical syndromes due to vitamin B_{12} deficiency are expected to be more frequent in the Indian population, the available evidence points to the contrary Nutritional megaloblastic anaemia seen in India is mostly due to folate deficiency [3, 5, 6] and neurological complications are conspicuously rare, even though the serum vitamin B_{12} levels in many individuals may be as low as in pernicious anaemia Further, serum levels of vitamin B_{12} may be even high in patients with frank protein caloric deficiency [19] and in protein-deficient rats [12]

It has been suggested that B_{12} deficiency may sometimes cause mental changes without accompanying haematological or neurological complications [8] However, no specific psychiatric syndrome could be attributed to such a deficiency and the vitamin B_{12} deficiency in these cases was diagnosed purely on serum determination and the results with B_{12} therapy equivocal Our previous studies on serum vitamin B_{12} levels in general population compared with psychiatric patients showed a similar frequency distribution [11] The Indian subjects in this study belonged to a higher socio-economic group and probably had low serum vitamin B_{12} levels since childhood without any apparent physical, haematological, or mental manifestations, they were all medical students Earlier, patients have been reported with low serum B_{12} levels but without anaemia, overt megaloblastosis or stigmata of B_{12} deficiency other than an increased lobe average of the nuclei of the neutrophils [14] However, in our subjects the neutrophil lobe count in non vegetarians did not differ significantly from that obtained in lactovegetarians It is difficult, therefore, to accept that all these lactovegetarians were abnormal simply because their serum vitamin B_{12} levels were lower than those of the non vegetarians

In a study on B_{12} -deficient megaloblastic anaemia a large proportion of 1 000 μg of injected hydroxycobalamin is retained as compared to normal subjects without anaemia [1] Furthermore, the liver B_{12} content in patients with B_{12} deficiency has been shown to be markedly low [2] If low serum vitamin B_{12} levels in our lactovegetarian subjects are indicative of over all tissue B_{12} deficiency then these subjects should have retained larger proportion of injected B_{12} in comparison with non vegetarians The studies with stable B_{12} mixed with labeled vitamin B_{12}, however, indicate that lactovegetarians excreted similar proportion of the injected dose as non vegetarians and the liver uptake of the two groups was also not significantly different Thus, the low serum vitamin B_{12} level may not necessarily indicate low tissue level and taken alone is not always diagnostic of B_{12} deficiency [13, 16]

The results of the urinary excretion of stable vitamin B_{12} revealed that lactovegetarians normally excreted significantly less vitamin B_{12} than the non vegetarians and the intestinal absorption of B_{12} in lactovegetarians was as efficient as in non vegetarians It appears that healthy lactovegetarians show some type of adaptation to low intake of vitamin B_{12} and the low serum vitamin B_{12} encountered in this group is not incompatible with normal health

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Acta haemat 53 98–104 (1975)

Lipid Peroxidation in Human Erythrocytes in Tocopherol Deficiency

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Abstract Lipid peroxidation following exposure to H_2O_2 vapour was abnormally great in the erythrocytes from certain patients with low plasma tocopherol concentrations Following treatment of such patients with tocopherol erythrocyte lipid peroxidation was markedly reduced The *in vitro* addition of tocopherol reduced lipid peroxidation in erythrocytes from both normal subjects and tocopherol deficient patients

Key Words
Erythrocyte metabolism
Lipid peroxidation
Malonyldialdehyde
Tocopherol

The erythrocyte membrane is rich in polyunsaturated fatty acids and these molecules are themselves highly susceptible to oxidation During its lifespan in the circulation the erythrocyte is exposed to small amounts of hydrogen peroxide generated as a result of normal metabolic processes, or to greater amounts resulting from the interaction of certain oxidant drugs and their metabolites with oxyhaemoglobin [6–8, 14] Intracellular H_2O_2 *may cause oxidation of haemoglobin resulting in Heinz body formation,* and also peroxidation of membrane lipids, with the formation of breakdown products including malonyldialdehyde (MDA) [1, 27, 28, 31] Detoxification of intracellular H_2O_2 is undertaken by the enzymes glutathione peroxidase and catalase [5, 11, 21, 23] A number of workers [12, 26, 29] have considered that tocopherol, in an antioxidant role, is probably involved in maintaining the erythrocyte membrane

It has been shown that erythrocytes from vitamin E-deficient rats underwent abnormally great haemolysis when exposed to dialuric acid, this haemolysis, which was associated with lipid peroxidation, was reduced by the addition of vitamin E (tocopherol) [4, 30] Similar results have been

obtained in several animal experiments in which H_2O_2 was used as the source of oxidant stress [12, 13, 19, 20, 29], and also in studies with erythrocytes from patients with abetalipoproteinaemia associated with tocopherol deficiency [9].

There has however been little investigation of the effect of tocopherol deficiency on the susceptibility to lipid peroxidation of human erythrocytes. We report such a study on erythrocytes from tocopherol-deficient adults, including observations on the effects of reducing the deficiency by tocopherol supplementation both *in vitro* and *in vivo*.

Subjects and Methods

Blood samples were obtained by venepuncture and collected in tubes containing lithium heparin. Normal subjects in this study were healthy medical and laboratory personnel. Studies were made on blood from patients who had been admitted to acute general medical wards at Maryfield Hospital, Dundee. Plasma tocopherol was measured by the method of BIERI and PRIVAL [2] and MDA by the method of STOCKS and DORMANDY [27]. Erythrocyte suspensions were prepared by washing blood twice in physiological saline and once in glucose-buffered saline. The cell suspension was then made up in glucose-buffered saline so that the haemoglobin concentration was 10–14 g%. Glucose-buffered saline was prepared by adding glucose at a concentration of 200 mg% to saline containing 0.01 M Sorenson phosphate buffer pH 7.0. Washed erythrocytes at a concentration of 4 g% haemoglobin were exposed to H_2O_2 vapour in the outer well of Conway units; hydrogen peroxide (1.8 ml of 8.8 M solution) was present in the inner well. Peroxidation of erythrocytes was carried out with a range of concentrations of the catalase inhibitor sodium azide added to the erythrocyte suspensions as previously described [31]. We have shown that supravital staining with crystal violet of erythrocytes previously exposed to H_2O_2 vapour resulted in the appearance not only of Heinz bodies, but also of an overall blue colouration of a proportion of the erythrocytes; a close relationship was found between the percentage of 'blue cells' and the degree of lipid peroxidation as measured by MDA formation [31]. In the present investigation the method of staining erythrocyte suspensions with crystal violet was as previously described [31]. Tocopherol, when required, was added to the erythrocyte suspensions in the Conway units, as α-tocopherol phosphate in saline, to make a final concentration of 5 mg%.

Results

Plasma tocopherol levels. In 50 normal persons the mean plasma tocopherol level (± SD) was 0.81 ± 0.18 mg%; the mean level in 21 male subjects (0.87 ± 0.17 mg%) was not highly significantly different from that

Table I The effect of added tocopherol on lipid peroxidation in normal erythrocytes, over a range of concentrations of sodium azide

	Concentrations of sodium azide, mmol/l				
	0	0 5	2 5	5 0	10 0
MDA, nmol/g Hb					
In the absence of tocopherol	9	58	140	181	256
In the presence of tocopherol	17	54	98	144	189
Blue cells, %					
In the absence of tocopherol	1	2	18	27	33
In the presence of tocopherol	1	2	14	18	25

for 29 females (0 77 ± 0 18 mg%) (0 1>p>0 05) A level of plasma tocopherol ≦0 50 mg% was regarded as indicating tocopherol deficiency

Lipid peroxidation in normal erythrocytes Washed erythrocyte suspensions from eight normal subjects were peroxidised in the presence of a range of concentrations of sodium azide, and lipid peroxidation was assessed by MDA and blue cell measurements At a concentration of 10 mmol/l azide, the mean MDA and blue cell formation (±SD) was 191 ± 34 nmol/g Hb and 31 8 ± 5 8%, respectively

In three experiments, the addition of tocopherol phosphate to normal erythrocyte suspensions peroxidised in the presence of azide resulted in a decrease in lipid peroxidation as measured by MDA and blue cell formation Typical results from one experiment are shown in table I

Lipid peroxidation in tocopherol deficiency. Erythrocyte suspensions *from 14 patients with tocopherol deficiency were peroxidised in the presence* of sodium azide (10 mmol/l) The diagnoses of this group of patients were post-gastrectomy (8), chronic pancreatitis (2), coeliac disease (2), Crohn's disease (1) and steatorrhoea of unknown cause (1) The mean values for MDA (236 ± 96 nmol/g Hb) and for blue cells (36 7 ± 14 7%) were not significantly different from those for the normal subjects, however, in five of the cases of tocopherol deficiency the results for MDA formation were 274–414 nmol/g Hb, that is, greater than 2 SDs above the mean of the normal group No apparent difference was found in the degree of tocopherol deficiency or in the clinical condition between these five patients and the other patients in the tocopherol-deficient group *Further studies were carried out in these five cases*

The in vitro effect of tocopherol on lipid peroxidation was studied in

Table II The effect of tocopherol on MDA formation in erythrocytes from tocopherol-deficient patients

Case	Age	Sex	Diagnosis	Plasma tocopherol mg/100 ml		MDA formation, nmol/g Hb		
				before therapy	after therapy	without tocopherol	tocopherol phosphate (5 mg/100 ml) added *in vitro*	following tocopherol therapy
1	62	F	coeliac disease	0.31	0.54	414	253	218
2	66	M	post-gastrectomy	0.40	–	405	336	–
3	47	M	post-gastrectomy	0.36	0.96	341	224	242
4	62	M	post-gastrectomy	0.24	0.83	286	184	193
5	56	M	chronic pancreatitis	0.33	0.92	274	238	304

Washed cells were exposed to H_2O_2 vapour in the presence of sodium azide (10 mmol/l)

erythrocytes from the five tocopherol-deficient patients referred to above. In each case, the addition of tocopherol resulted in a marked reduction in MDA formation (table II), which became less than 2 SDs above the normal mean in 4 of the 5 cases.

The effect of tocopherol therapy. Four of the above tocopherol-deficient patients were treated with α-tocopherol acetate (Roche), 100 mg intramuscularly daily for 3–5 days. MDA formation was measured before the therapy and 2 days after the last injection. Washed cells were exposed to H_2O_2 vapour in Conway units in the presence of sodium azide (10 mmol/l) as described above. In 3 of the 4 cases (No. 1, 3, 4), there was a marked reduction in MDA formation following tocopherol therapy (table II). In case 5, there was no immediate decrease in lipid peroxidation following treatment; however, when erythrocytes from this patient were studied again several months later, the value for MDA had fallen from 274 to 149 nmol/g Hb. The significance of this latter finding is discussed.

Discussion

Several drugs, including antimalarials of the 8-aminoquinoline group, certain sulphonamides and nitrofurans, are known to exert an oxidant effect on the erythrocyte [8]. This oxidant stress results from the intracellular generation of hydrogen peroxide by an autoxidation of the drug with oxyhaemoglobin [6, 7, 14]. The relative importance of tocopherol and the enzymes glutathione peroxidase and catalase in erythrocyte antioxidant protection is difficult to evaluate. In this study the greatest difference in lipid peroxidation between normal subjects and tocopherol-deficient patients was found at the highest concentrations of the catalase inhibitor sodium azide. This implies that in the circulating erythrocyte, tocopherol acts as a second line of defence to the H_2O_2-detoxifying enzymes in protecting the cell from the effects of peroxidation.

Patients with alimentary tract disorders associated with impaired absorption of tocopherol may also suffer from other nutritional disorders. In this investigation the role of this vitamin in preventing lipid peroxidation was studied by the addition of tocopherol both *in vitro* and *in vivo*; tocopherol resulted in a marked decrease in lipid peroxidation as measured by MDA formation. Erythrocytes from one patient treated with tocopherol did not immediately show a decreased tendency to lipid peroxidation, although this did become apparent when further studies were made several months later. This may be the result of a variable rate of uptake of tocopherol from the plasma by the erythrocyte. As the cell suspensions in these experiments were previously washed, it is the effect of tocopherol which has been taken up by the cell that is being measured.

The importance of an adequate tocopherol status in man may be seen from the reports of haemolytic anaemia in certain premature infants with tocopherol deficiency [16–18, 22, 25, 33] and in a reduced red cell lifespan in adults with tocopherol deficiency as a result of malabsorption [3, 10, 15, 24]. The possible relevance of tocopherol deficiency to the anaemia of patients with malabsorption remains to be clarified. We have recently shown [32] that erythrocytes from adult patients with tocopherol deficiency show increased susceptibility to Heinz body formation when challenged by oxidant stress. It therefore appears probable that increased susceptibility to oxidation of both haemoglobin and the membrane lipids contribute to the haematological effects of tocopherol deficiency.

Acknowledgements. The technical assistance of Miss JOAN SMITH is gratefully acknowledged. We are indebted to Roche Products Ltd. for the supply of ampoules of

α-tocopherol acetate. This research programme was supported by a grant from the Scottish Hospital Endowments Research Trust.

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Acta haemat 53 105-108 (1975)

Aplastic Anaemia after Exposure to a Weed Killer, 2-Methyl-4-Chlorphenoxyacetic Acid

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Key Words
Aplastic anaemia
2 methyl-4-chlorphenoxyacetic acid
Pancytopenia
Weed killers

Abstract A 64 year-old farmer developed aplastic anaemia after exposure to 2 methyl-4 chlorphenoxyacetic acid while spraying weed killer Muscular weakness, haemorrhagic gastritis and slight signs of liver damage occurred at the same time All these symptoms, including blood dyscrasia are consistent with those described as toxic effects of chlorphenoxyacetic acids in animal experiments A causal relationship between aplastic anaemia and the 2 methyl-4-chlorphenoxyacetic acid thus seems probable The anaemia was reversible but the case serves as a warning that careful safety measures are required during the use of chlorphenoxyacetic acids and related compounds

Many cases of aplastic anaemia remain 'idiopathic', as no specific causal agent can be identified Among the known agents causing aplastic anaemia, benzene is the first known Some derivatives of benzene have this same property, hexachlorbenzene (Landan) being perhaps the best known [1, 4, 5] Another compound containing a halogenated benzene ring is chlorphenothane (DDT) which is also a cause of aplastic anaemia [1, 4] These compounds are widely used as insecticides, and all the cases of aplastic anaemia attributed to them are in connection with exposure in agriculture

Further chemicals containing a halogenated benzene ring and extensively used for spraying are the chlorphenoxyacetic acids, which are employed as weed killers Recently, we detected a case of aplastic anaemia after exposure to 2 methyl-4-chlorphenoxyacetic acid (MCPA)

Case Report

A 64 year old farmer visited his local health officer complaining of weakness He was found to be anaemic and was referred to hospital in September 1973 He had a history of coronary heart disease extending back several years and regularly took digoxin and nitroglycerin tablets During the last 3 years he had not taken any drugs known to cause aplastic anaemia

During the last 5 years he had sprayed chemicals to destroy weeds on his farm In the previous June and July he had been spraying Hormotuho X Super® (Kemira Ltd, Helsinki) a compound containing MCPA He employed an old manual spraying device with the container on his back The container was leaking so that his clothes were wet with spraying solution Two weeks after the last spraying he detected *spontaneous haematomata on his body and began to feel lethargic The first* visit to a doctor was however delayed by 2 months as he thought the symptoms as *occasional ones*

On admission he was pale the tourniquet test was positive but otherwise the physical findings were normal Haemoglobin 8 4 g%, leucocytes 16 300 /µl with 48% neutrophils platelets 127 000/µl and reticulocytes 2 4% The bone marrow morphology was normal His serum bilirubin transaminases alkaline phosphatase glutamyl transferase and ornithine carbamyl transferase were normal but the thymol turbidity test was slightly pathological (0 175) Serum iron was 32 µmol/l TIBC 40 µmol/l, B_{12} 700 pmol/l and folate 10 5 mmol/l The urine sediment was normal and tests for glucose and protein negative On gastroscopic examination haemorrhagic gastritis was found

During the subsequent weeks he became more anaemic and received several transfusions of packed red cells His lowest blood values were Hb 7 3 leucocytes 2 400 with 44% of neutrophils and platelets 37 000

He was treated with prednisolone (80 mg daily orally) with no haematological response for 10 days then methenolon (Primobolan® Leiras/Schering Turku 100 mg daily) was added and the dosage of prednisolone gradually withdrawn to 15 mg daily Two months later his blood count was Hb 12 6 leucocytes 5 100 platelets 308 000 serum iron 6 8 Five months later he was free of symptoms and had Hb 13 5 leucocytes 7 600 with 46% of neutrophils and platelets 190 000

Discussion

A panmyelopathic blood picture developed after exposure to MCPA in a man without any previous haematological history, intake of drugs, or exposure to chemicals hitherto known to cause aplastic anaemia Exposure to the same compound or related compounds had already occurred during the previous 4 summers There had thus been an opportunity for sensitization against the chlorphenoxyacetic acids On the other hand, the exposures have been massive, as all the safety measures had been neglect-

ed. On the contrary, a severely leaking device had been employed for spraying. Hence, a direct toxic effect is possible.

In animal experiments using dogs, feeding large doses of 2,4-dichlorphenoxyacetic acid (2,4-D) have resulted in loss of appetite, apathy, general muscular weakness, and after convulsions death in a coma or ventricular fibrillation [3]. In some cases, irritation and bleeding of the mucous membranes has been detected. Necropsies after suicides with phenoxyacetic acids have revealed inflammation, necroses and ulcerations of the digestive tract, necroses of liver and degenerative changes in the renal tubuli [2]. Of these symptoms, haemorrhagic gastritis, a tendency for bleeding, muscular weakness and slight liver damage were present in our case. The clinical picture thus fits with that of poisoning by a phenoxyacetic acid. In our case, at least thus far, all the symptoms and damage have been reversible.

As far as any causal relationship between exposure to MCPA and aplastic anaemia is concerned, a provocation test would, of course, supply convincing proof. As this cannot be done, more indirect evidence must be considered. Some chemical compounds related in their structure to MCPA, that is compounds with a halogenated benzene ring, are known to cause aplastic anaemia [1, 4, 5]. In addition, subacute intoxication of dogs with 2,4-D has resulted in anaemia, leukopenia and thrombocytopenia [3]. The time relationship between the exposure to MCPA and the onset of the symptoms is in accordance with the possibility of a causal relationship between the chemical and anaemia. Especially should the cumulative action of smaller doses of chlorphenoxyacetic acids be kept in mind [3]. It is thus very likely, that this aplastic anaemia was indeed caused by MCPA. Although the blood dyscrasia appeared at first only in the form of anaemia, pancytopenia of all the myeloid cell lines later developed.

Our patient had neglected all the safety measures when spraying MCPA weed killer preparations, having thus laid himself open to massive exposure to the chemical. This example again points to strict safety measures in order to minimize exposure to such agents as insecticides and weed killers.

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Acta haemat 53 109 117 (1975)

New Kind of Cytoplasmic Inclusions of Plasma Cells in Acid Maltase Deficiency[1]

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Abstract Extensive vacuolization could be demonstrated in nearly all plasma cells and in some lymphocytes of an adult with glycogenosis type II (Pompe s disease) The biochemically defined diagnosis acid maltase deficiency (AMD) could be ascertained by examination of the maltase activity of the patient s leukocytes. Electron microscopical microspectrographic, and cytochemical investigations revealed electron dense inclusions, which show an UV absorption at 276 nm and a positive reaction after PAS staining of plastic embedded material Since no other abnormalities of the plasma cells could be found our results are presumably indicative for a connection of AMD and a glycoprotein storage in the plasma cells of the patient

Key Words
Acid maltase deficiency
Electron microscopy
Glycogenosis
Leukocyte maltase
Plasma cell inclusions
Pompe s disease

In an adult with acid maltase deficiency (AMD) associated with advanced muscular dystrophy (glycogenosis type II, Pompe s disease) we have regularly found plasma cells with many vacuoles in bone marrow samples after panoptical staining and isolated lymphocytes containing vacuoles in the peripheral blood To our knowledge such cytoplasmic abnormalities appearing extensively in nearly all plasma cells have not until the present time been described in connection with a metabolic disorder – particularly AMD – or in connection with any other disease We will report our biochemical, light and electron microscopic, microspectroscopic and cytochemical examinations in this patient

[1] Supported by the Deutsche Forschungsgemeinschaft

Material and Methods

Material Bone marrow biopsies from the patient of the sternum and pelvis blood smears and leukocyte concentrates were done without the addition of anti coagulant The tests were also carried out on a healthy brother of the patient

Staining methods for light microscopy May Grünwald Giemsa methylene blue pH 9 methyl green pyronin Adams reaction Sudan black B oil red O PAS and PAS after pretreatment with lead acetate as an oxidant and after incubation with amylase and hyaluronidase [2] as well as methods for the determination of non specific esterase [15 16] acid phosphatase [15 16] *β* glucuronidase [10 17] and peroxidase [13] Methylene blue methyl green pyronin and PAS were also carried out on 1 *μ*m thick plastic embedded sections after removal of the embedding ma terial [20]

Microspectrophotometry Another part of the material was smeared on quarzc slides and air dried The smears were examined after mounting in glycerol with a double beam microspectrometer (US 1206 Beckman Instruments Munich FRG) The measuring arrangement employed allowed a point determination of a single inclusion of 2 *μ*m cross section against a reference beam but also the integrat on of more inclusions in a larger field

Electron microscopy Fragments of a sternal puncture sample were fixed in 2% glutaraldehyde in 0 135 M phosphate buffer pH 7 2 for 3 h and a part was post fixed in 1% osmiumtetroxid in the same phosphate buffer After washing in buffer the preparation was dehydrated through an acetone series and embedded in Aral dite (Durcupan AMC Fluka Switzerland) in the usual way Serial ultra thin sec tions were cut on an ultramicrotome (Reichert OM U_2) 1 *μ*m thick sections were prepared for light microscopy The ultra thin sections (silver interference colour) were picked up on a slot grid (Veco L 12) that had been covered with formvar and carbon [11] The electron m croscopic pictures were taken on a Siemens El miskop 101 and a Philips 201 35 mm Agfa rollf lm 220 and Kodalith LR film 2572 were used

Leucocyte maltase activity 40 ml of venous blood were taken into citrate from the patient and five controls carried out in order to isolate the leukocytes After the addition of one fifth of the volume of 5% dextran the erythrocytes were re moved by a Ronpaquon dextran dense medium [5] The following steps were car ried out at 0 °C The leukocytes were collected from the plasma by centrifugation and taken into 2 ml 50 mM sodium fluoride The cells were disrupted four times by ultrasonication for 12 sec intervals at 100 W with a Branson B 12 ultrasonica tor Nuclei and remaining undisintegrated material were removed by centrifuga tion at 1 200 *g* for 10 m n Protein determination was carried out by the method of LOWRY *et al* [18] The maltase activity was determined at different pH values beginning with pH 4 0–5 5 in 70 mM acetate buffer and from pH 5 5 to 7 0 in 70 mM cacodylate buffer with 50 mM maltose as substrate and with an average ad dition of 0 32 mg dialyzed protein per assay volume of 0 4 ml The incubation was carried out at 37 °C for 3 h The reaction was stopped by the addition of 0 1 ml 1 N perchloric acid at 0 °C [1] The quantity of glucose formed in this assay was determined from 0 1 ml of the resulting supernatant with the hexokinase and glu cose 6-phosphate DH reaction [4]

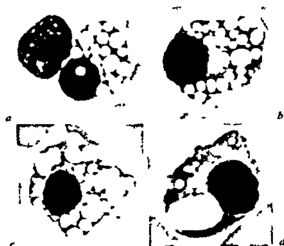


Fig 1 Bone marrow smears. May-Grunwald-Giemsa stain Three plasma cells with small to medium sized vacuoles (a, b c) one with a very large vacuole indenting the nucleus and five small vacuoles (d) × 1 400

Results

Light Microscopy

In the examined smears which had been taken at three different times from the sternum and pelvis, a pronounced vacuolization of more than 90% of the plasma cells was found the total number of plasma cells being in the normal range The vacuoles were preponderately round and measured 0 2–4 0 μm cross section, a few were greater reaching the size of erythrocytes Usually the whole cytoplasm contained additionally numerous small vacuoles (fig 1) In EDTA free blood smears isolated lymphocytes – less than 5% – were noticed, that had vacuoles in the cytoplasm In spite of different fixation methods for smear preparations no inclusions were detected With the clinically healthy brother of the patient the described changes were not observed

Microspectrophotometry

This examination gave a first hint for the existence of inclusions by means of light absorption with a sharp narrow peak at 276 nm The microspectrographic exam nations showed for the isolated measurement of a single vacuole' an absorption at 276 nm Between 300 and 600 nm an

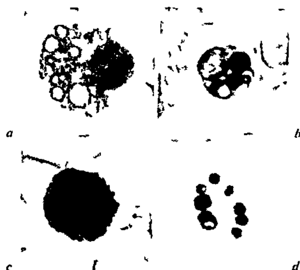


Fig 2 *a* Acid phosphatase reaction (naphthol AS biphosphate hexazonium pararosanilin pH 5 0) The activity is confined to the border of the vacuoles and the cytoplasm *b* Methylene blue stain pH 9 0 Plastic embedded section showing large inclusions within a plasma cell *c* Blood lymphocyte with a very intense granular PAS positivity *d* Semi thin section of plastic embedded bone marrow, PAS stain Inclusions in plasma cells are positive

irregularity of the transmission curve is evident that can be explained by optical effects The integrated measurement of many inclusions shows a similar absorption at 276 nm An irregularity occurs at 400–450 nm, which is presumably caused by the cytoplasm and its organelles

Cytochemical Examinations

Smears With all reactions, which serve to identify the material in the 'vacuoles', they seemed to be optically empty The reaction for peroxidase was negative The demonstration of acid phosphatase activity showed a strong reaction at the rim of the vacuoles besides the usual activity in the vacuole-free cytoplasm (fig 2a) The same behaviour is found for β-glucuronidase, whose activity appeared weaker Esterase activity was confined to the vacuole-free cytoplasm An intense granular PAS reaction corresponding with a diffuse weak PAS reaction in the cytoplasm of some plasma cells between the vacuoles – most evident in those cells with less vacuoles – was demonstrable, similar to an extraordinarily intense granular reaction in some lymphocytes of the peripheral

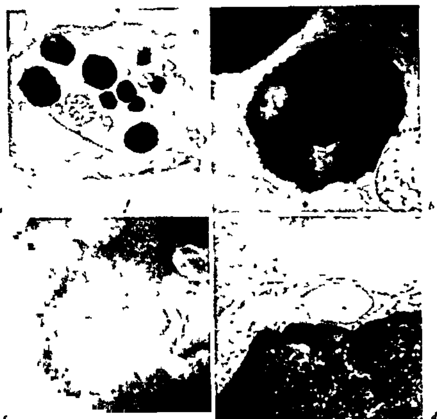


Fig. 3. Electron micrographs of plasma cells containing inclusions. a Typical arrangement of endoplasmatic reticulum and multiple small vacuoles near to the border of the cell. Besides there are electron-dense inclusions with different shape and consistency and three honeycombed inclusions. × ca. 10×10³. b Electron-dense inclusion with myelin-like areas and polycrystalline structure. In the lower right corner part of a honeycombed inclusion. × ca. 40×10³. c Detail of Figure 3b showing the polycrystalline structure with a periodicity of 100 Å. × ca. 5×10⁴. d Border of an inclusion with small homogeneous areas with a lighter centre in the case [illegible]. × ca. 40×10³

blood and bone marrow, not observable in this degree normally or in leukemia-free cases (Fig. 2c). After treatment with amylase the reaction was weaker.

Staining. In plastic-embedded semithin sections the vacuoles after methylene blue staining at pH 9 were mainly identifiable as round, [illegible]

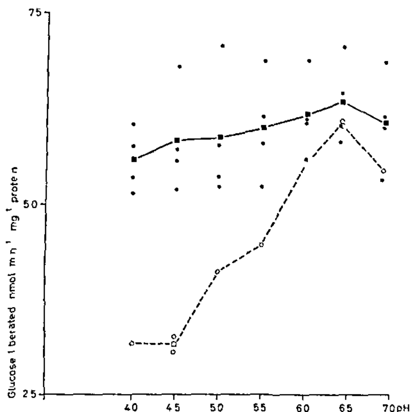


Fig 4 Specific activity of leukocyte maltase at different pH values The patient's leukocytes (○) Leukocytes of four controls (●)

bly intense and homogeneously stained inclusions, whether the embedding material was left or dissolved (fig 2b) These inclusions were PAS-positive After pretreatment with amylase the reaction was only minimally reduced It remained positive if the oxidation with periodic acid was replaced by leadtetraacetate (fig 2d)

Electron Microscopy

Electron microscopy (EM) showed the plasma cell inclusions to be of variable density to electrons The inclusions appeared round to polygonal with fine granules, sometimes, however, honeycombed and on rare occasions empty These different varieties of inclusions were found side by side in one cell (fig 3a, b) The most electron dense, which appeared homogeneous at low magnifications, showed a polycrystalline structure with a periodicity of approximately 100 Å (fig 3c) Besides this, myelin-like areas in the inclusions were sometimes found (fig 3b) The inclu-

sions lay between the membranes of the endoplasmatic reticulum. On the border of the inclusions were occasionally found small homogeneous spots which showed in some cases a lighter centre (fig. 3d). A defining membrane of the inclusions could not be identified.

Maltase Activity in Leukocytes

The deficiency of acid maltase could be demonstrated by biochemical analysis of leukocytes. The pH dependency of maltase activity of leukocyte extractions from the patient as well as from five controls are shown in figure 4. It can be seen that the leukocyte enzyme from the patient has only low activity in acidic conditions. The displayed curve is very comparable with those mentioned of other patients with AMD in the literature [8].

Discussion

A Persian student with AMD, whose diagnosis was confirmed by means of biochemical examination of his leukocyte maltase activity, was found to have a pronounced vacuolization of the plasma cells from the bone marrow. Moreover, a heavy deposit of PAS-positive material was visible between the vacuoles of some plasma cells and particularly pronounced in the lymphocytes of the peripheral blood and bone marrow. These vacuoles, which appeared optically empty in smears, contained inclusions in semi thin sections of plastic embedded material and also in EM preparations. They were stainable by the PAS reaction and with methylene blue pH 9 and showed an absorption maximum at 276 nm with UV microspectrography. EM revealed electron dense inclusions which were in part polycrystalline and sometimes associated with myelin structures and in part varied from honeycombed to completely empty. Our results gave evidence that they consist of both protein and polysaccharide components.

Since our patient's total serum protein and serum electrophoresis along with the quantitative determination of the immunoglobulins gave values in the normal range and the immunoelectrophoresis including the application of anti μ-chain antibodies showed no abnormal protein fraction [9], plasma cell alterations with dys- or paraproteinemia could be discounted.

In publications about AMD in children and in few communications concerning the disease in adolescents and adults an abnormality of the

plasma cells has not been previously described Only ENGEL and DALE [7] have mentioned isolated vacuolized lymphocytes in the peripheral blood such as have also been seen in amaurotic idiocy Tay-Sachs, in Niemann-Pick disease, and in progressive muscular dystrophy type ERB (Jordan's anomaly) [3, 12, 23] In addition, however, isolated vacuolized lymphocytes are found in various diseases They can in any event only serve as a hint to look for a metabolic disorder

In AMD glycogen-rich, membrane enveloped inclusions were known in the muscle fibres and in fibroblasts after tissue culture until now [6, 7, 19, 21] In contrast, descriptions of plasma cells containing inclusions of glycoprotein have not been given previously

Our results show a clear-cut difference of the inclusions described in this paper from other examples in particular from Russell bodies by means of EM Indeed such a pronounced vacuolization of all plasma cells is known neither in connection with any other muscular disease or metabolic disorder nor another disease We therefore presume that the inclusions are connected with AMD We must leave open the question whether the special protein synthesizing and secretory mechanisms of plasma cells cause the laying down of protein polysaccharide complexes in the described form, whereas in other sites (e g muscle) a concentration of glycogen in secondary lysosomes is formed [7, 21]

Acknowledgements We are greatly indebted to Prof Dr F ERBSLÖH, Director of the Department of Neurology of the Justus Liebig Universität Giessen who referred the patient to us

We would also like to thank Dr G HARTWIG Center of Anatomy and Cytobiology, for his friendly cooperation

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Acta haemat 53 118–127 (1975)

Classical Factor X Deficiency

Report of a Further Case[1]

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Abstract A case of classical factor X deficiency is reported The propositus is a 28 year old male who presented easy bruising, epistaxis, hematomas, hematuria and occasional hemartrosis since early childhood The severely prolonged prothrombin time was corrected by normal serum but not by adsorbed normal plasma The abnormality was not corrected by the plasma of a patient with factor X deficiency, but by the plasma of patients with factor II or VII deficiencies Partial thromboplastin time prothrombin consumption and the thromboplastin generation test were abnormal The thromboelastogram showed a prolonged 'K' and 'r' together with a normal ma' Factor X was very low (<1%) Platelet tests were normal No factor X band or precipitates were seen on electroimmunoassay and on the cross over electrophoresis The non consanguineous parents and several other members of the family were found to be heterozygotes

Key Words
Bleeding disorders
Blood coagulation
Electroimmunoassay
Electrosyneresis
Factor X deficiency

Classical congenital factor X deficiency remains a very rare coagulation disorder To date only about 25 proven or probable cases have been reported in the literature [2–4, 6–8, 14, 19, 21, 23, 25–31] The reasons for such rarity are unknown and seem really striking if one takes into account the fact that congenital factor VII deficiency, a condition which resembles factor X deficiency, is at least 4 times more frequent [16]

Recently several patients with a coagulation disorder due to the presence of an abnormal factor X have been described [13, 14, 15, 17] This

[1] This study was supported in part by a grant from the CNR (grant CT 73 00599 04)

variant of the factor X defect has been termed factor X Friuli since the patients originally described came from an isolated valley of the north-eastern Italian region called Friuli The main feature of factor X Friuli patients lies in the presence of an abnormal factor X, a protein which may not be activated, or only very slowly so, by tissue thromboplastins, whereas it may be normally activated by Russell's viper venom Consequently, in these patients, factor X is found low only when assayed using tissue thromboplastins However, even taking into account all the Friuli patients, the total number of patients with a factor X defect or abnormality does not exceed the total of 40

Reports of new cases with classical factor X deficiency should be welcome since they could help clarify the main features of the disease The object of the present paper is to present a new patient with this condition The propositus is the fourth patient with this coagulation defect reported in Italy [2, 14, 27]

Material and Methods

Materials and methods have been described elsewhere [11-14] Only new data will be given herein

Prothrombin consumption was carried out after addition of normal serum to the patient's serum in a 2 10 proportion Factor II in the one stage or two-stage system was assayed after the addition of an aliquot of normal serum to the factor II substrate the patient's defibrinated plasma or the diluted thromboplastin and patient's plasma mixture The antiserum used for immunological studies was kindly supplied by Dr D Aronson of the National Institutes of Health Bethesda, Md [1] Electroimmunoassay was carried out according to the method proposed by Laurell [24] The antiserum used in this system was absorbed with factor X deficient plasma as previously reported [15 18] Cross-over electrophoresis (electrosyneresis) was carried out according to a previously reported modification [15] of the method proposed by Bussard [5] without absorbing the antiserum

Case Report

The propositus is a 25 year-old male who was first seen by one of us (P C) in March 1974 when he was admitted to the Bolzano City Hospital because of a hematoma of the left thigh Routine coagulation studies and cross-correction studies with normal serum and absorbed normal plasma raised the question of factor X deficiency

Subsequently further studies done in Padua confirmed the diagnosis. The parents of the propositus were not consanguineous but were born in the same valley of the Upper Adige region Family history was negative for severe bleeding manifestations (fig 1)

■ = Propositus homozygote symptomatic
◧ or ◐ = Male or female heterozygote asymptomatic or mildly symptomatic
⊟ or ⊖ = Deceased asymptomatic normal ?
◫ or ⦶ = Not studied symptomatic normal ?
□ o O Studied normal

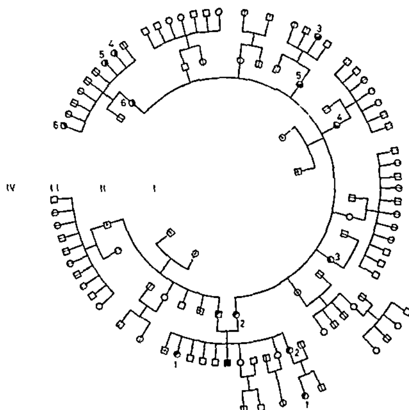


Fig 1 Family pedigree Eleven persons besides both parents were found to be heterozygotes The factor X levels found in the normal relatives varied between 86 and 125% Those found in the heterozygote relatives varied between 47 and 74% (see table IV)

The patient started to present easy bruising and hematomas after accidental falls or major traumas in early childhood Epistaxis and hematuria appeared too at this age At the age of 8 the patient was admitted to the Bolzano City Hospital for anemia On that occasion there were no bleeding manifestations but the patient was transfused with 3 units of blood to correct the anemia At the age of 12 the patient was admitted to a local hospital for a huge hematoma of the right thigh and on another occasion for severe epistaxis In 1963 the patient had to be admitted again to a local hospital for anemia and exertion dyspnea On that occasion he received several blood transfusions In 1964 the patient was admitted to the University Hospital in Innsbruck were a tentative diagnosis of factor X deficiency was apparently first postulated In 1965 the patient was admitted to a local hospital for post transfusion

Table I. Coagulation study in the propositus

Test	Propositus' values	Normal values
Glass clotting time, min	16	5-9
Platelet count	370 000	150 000–350 000
Clot retraction	complete after 6 h	complete after 12 h
Bleeding time, min	4.5	2–5
Partial thromboplastin time, sec	144	35–45
Prothrombin time, sec	125	14–15
Stypven-cephalin clotting time, sec	57	12–14
Thromboplastin generation test	58 sec in 8 min	15 sec in 6 or 8 min
Prothrombin consumption, %	48	90
Fibrinogen, mg%	380	250–450
Factor II (one-stage), %	96	85–125
Factor II (two-stage), %	100	85–125
Factor VII, %	100	85–125
Factor X, %[1]	<1	85–125
Factor V, VIII, IX, XI, XII	normal	60–150%
Thrombin time, sec	19	18–25
Thromboelastogram, mm		
'r'	71	10–20
'K'	55	6–12
am	52	50–66

[1] Same values with tissue thromboplastin and Stypven-cephalin

hepatitis. From 1964 till 1973 the patient had to be admitted on several occasions to local hospitals for spontaneous or post traumatic hematomas and melenas. Hemarthroses have been rare but have occasionally caused temporary limitations of mobility. However, no permanent ankylosis has occurred.

Results

The coagulation study is summarized in table I. Our data clearly indicate that the propositus had a first- and second-stage coagulation defect. This is consistent with factor X deficiency once a factor V or factor II deficiency is ruled out.

The prothrombin time is corrected by normal serum, normal plasma, factor II- and factor-VII-deficient plasma but not by adsorbed normal plasma, factor-X-deficient plasma and the abnormal *factor X (factor X Friuli)* plasma (table II). Prothrombin time derivative tests such as PP test, thrombotest and normotest were severely prolonged too (table III).

Table II Prothrombin time correction studies in the propositus Mixtures of equal parts

	Prothrombin time, sec	
	mixture	reference plasma
Patient's plasma alone	125 0	–
Patient's plasma + normal plasma	16 1	14
Patient's plasma + normal serum	15 8	–
Patient's plasma + adsorbed normal plasma	102	–
Patient's plasma + plasma of another patient with factor X deficiency[1]	114 2	111
Patient's plasma + plasma of a patient with factor X Friuli coagulation disorder[2]	42 8	35
Patient's plasma + plasma of a patient with hypoprothrombinemia[1]	15 4	20 5
Patient's plasma + plasma of a patient with factor VII deficiency[1]	16	50 1

[1] Personal case, frozen plasma
[2] Personal case (index patient with factor X Friuli disorder)

Table III Prothrombin time derivative tests in the propositus

Test	Propositus' values	Normal control
PP test, sec[1]	306 0	28
Trypsin clotting time, sec	86 0	23 3
Thrombotest, sec	706	43
Normotest, sec	405	25 3

[1] Tissue thromboplastin is used in the system

The factor X level was less than 1% regardless of the assay system Factor II was normal both by the one-stage and the two-stage method

Factors V, VII, VIII, IX, XI and XII were all within normal limits Fibrinogen and fibrinolysis were normal too The prothrombin consumption was defective The thromboplastin generation test was abnormal too and the substitution of the patient's serum with normal serum corrected the abnormality (fig 2) The thromboelastogram showed a clearly prolonged 'r' and 'K', whereas the 'ma' was normal Thrombin time and platelet tests were all within normal limits Immunologically, no factor X peak was evident in the patient plasma on electroimmunoassay and no

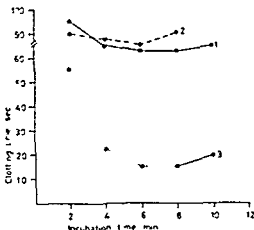


Fig 2 Thromboplastin generation test 1 -- Generation mixture containing patient's adsorbed plasma and patient's serum 2 -- generation mixture containing adsorbed normal plasma and patient's serum 3 -- generation mixture containing patient's adsorbed plasma and normal serum In each case the other components of the generation mixture were an activated cephalin preparation and 0 025 M $CaCl_2$

band was seen on electrosyneresis (fig 3, 4) Both parents of our propositus together with other family members were found to have slightly reduced factor X levels (47–74%) and were considered to be heterozygotes (fig 1 table IV)

Discussion

The main diagnostic criteria for classical factor X deficiency are prolonged prothrombin time corrected by serum prolonged partial thromboplastin time and Stypven-cephalin clotting time and abnormal thromboplastin generation due to a seric defect Our patient fully meets all these criteria The lack of correction of the propositus' prothrombin time after the addition of known factor X-deficient plasma firmly established the diagnosis The type of deficiency is the classical type in the sense that factor X was low in both coagulation assay systems (tissue thromboplastin and Russell's viper venom) and immunologically

In the factor X Friuli coagulation disorder factor X is found to be low only when tissue thromboplastin instead of RVV-cephalin is used in the assay system [13 14 16 17] and a normal level is obtained immunologi

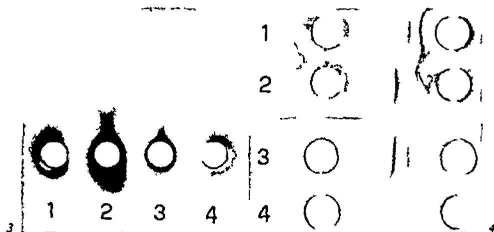


Fig 3 Laurell electroimmunoassay 1 = Propositus plasma 2 = abnormal factor X (factor X Friuli) plasma 3 = pooled normal plasma 4 = Dade factor X deficient plasma No peak is seen in 1 and 4 The antiserum used in these studies was absorbed with factor X deficient plasma

Fig 4 Cross over electrophoresis (electrosyneresis) 1 = Propositus plasma 2 = pooled normal plasma 3 = abnormal factor X (factor X Friuli) plasma 4 = Mr Stuart plasma The antiserum used in these studies was not absorbed A major or factor X band is visible only in 2 and 3 Lighter or minor bands are present in all plasmas but they may be disregarded since they are due to secondary activities of the antiserum used

cally The severely prolonged prothrombin time presented by our propositus is of interest The value obtained is longer than the value reported for Mr Stuart (about 85 sec) [23] Only 2 other patients with factor X deficiency had similarly prolonged prothrombin times namely a patient presented by LECHLER *et al* [25] in 1965 and a patient studied by us in 1970 [4] The bleeding manifestations presented by our propositus were severe *and were those common in classical factor X deficiency The se*verity of the bleeding manifestations correlates well with the very low factor X level In this regard it is interesting to note that in general bleeding manifestations are more severe in the classical factor X deficiency than in the abnormal factor X (factor X Friuli) coagulation disorder [17]

As far as heredity is concerned there is no doubt that our patient was a homozygote for the defect Both parents were in fact heterozygotes Eleven relatives of the propositus besides his parents were also heterozygotes for the abnormality The hereditary data gathered from this study fully confirm the previous observations on the matter [20 29] The defect is transmitted as an autosomal incompletely recessive trait The heterozy

Table II. Factor X level and bleeding manifestations in the heterozygote family members

Position in family tree (fig. 1)	Factor X, %	Bleeding manifestations
II_1	70	none
II_2	55	excessive bleeding after hysterectomy, epistaxis
II_3	60	none
II_4	74	none
II_5	72	none
II_6	60	none
III_1	50	none
III_2	60	none
III_3	60	excessive bleeding after tooth extractions
III_4	58	none
III_5	47	excessive bleeding after tooth extractions
III_6	68	none
IV_1	60	none

Table V. Main genetic variants of factor X defect. Our propositus has a coagulation pattern identical to that shown by Mr. Stuart

Index patient	Factor X level (tissue thromboplastin)	Factor X level (Stypven-cephalin mixture)	Factor X antigen
Miss Prower	low	low	normal
Mr. Stuart	very low	very low	absent
Factor X Friuli	low	normal	normal

Very low = ≤1%, low = 2–15%, normal = 70–100%

gote subjects show factor X levels varying usually between 40 and 70% of normal and are often asymptomatic. The abnormal factor X (factor X Friuli) coagulation disorder has the same hereditary transmission [12, 13]. The prothrombin time in the heterozygote population may be normal or slightly prolonged. In the present family the prothrombin time was 1–1.5 sec prolonged in almost all heterozygotes. Factor X levels varied between 47 and 74%. A few of the heterozygotes belonging to the present pedigree have presented mild and minor bleeding manifestations (excessive bleeding after tooth extractions and epistaxis) (table IV).

Three genetic variants of the factor X defect seem to exist today (table V). Mr. Stuart has very low factor levels regardless of the method used

and no factor X antigen, Miss Prower has low factor X level in both assay systems but normal factor X antigen [9] The index patient with the factor X Friuli abnormality has low factor X level in the tissue thromboplastin assay system whereas a normal level is obtained in the Stypven cephalin assay and immunologically Our propositus has a defect identical to that shown by Mr Stuart namely classical factor X deficiency

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Materials and Methods

Clinical series The series consisted of 78 healthy newborn infants, aged 1 h to 12 days at the time blood was taken, and all delivered at the Women's Clinic, University of Helsinki Their gestational age varied between 33 and 43 weeks The first Apgar score of the infants ranged from 7 to 10 and the birth weight in each case was over 2,500 g 25 of the infants were delivered by Caesarean section because of maternal dystocia Samples of cord blood were taken from an additional 12 full term infants

Determination of cell proliferation in the blood Cell proliferation in the cord blood was determined as described elsewhere [13] To study the capillary blood of newborn infants a micro method was used 50 µl of blood, obtained from the heel with a capillary pipette, was transferred into a prewarmed (37 °C) centrifuge tube that held 0 5 ml medium RPMI 1640 containing 20% calf serum (CS) 50 IU/ml heparin and 2 µCi/ml tritiated thymidine (^{3}H TdR, specific activity 6 7 µCi/mM), and then incubated for 30 min at 37 °C. After incubation 0 1 ml of the suspension was diluted with 0 9 ml of 2% acetic acid, and nucleated cells were counted in a haemocytometer To separate the mononuclear cells the rest of the suspension was layered on top of 1 ml of Ficoll Isopaque mixture in a narrow centrifuge tube and spun at 400 g for 20 min [4] The interphase cells were collected, washed with 5 ml of RPMI 1640 containing 20% CS, and then resuspended in 0 5 ml of the same medium Cell preparations for May Grünwald Giemsa staining and for autoradiography were made with a cytocentrifuge Autoradiographs were prepared with Kodak AR 10 stripping film exposed for 5–6 days and stained with Giemsa

The percentages of labelled lymphoid cells (LLC) labelled myeloid cells (LMC) and labelled erythroid cells (LEC) were determined morphologically as described elsewhere [13, 21] Differential counts of white blood cells were made from regular blood films prepared the time each sample was taken

Rosette formation 2 ml of heparinized (50 IU/ml) blood, taken from a superficial cephalic vein, was diluted with 6 ml of phosphate buffered saline (PBS) and incubated in an Erlenmeyer flask with 0 2 g Carbonyl iron powder (General Anilin Corporation New York, N Y and 1 µCi/ml ^{3}H TdR for 1 h at 37 °C Every 10 min the flask was gently agitated After incubation the iron and the cells that had phagocytosed iron were removed from the suspension with a Teflon-coated magnet and the mononuclear cells that remained were separated by the Ficoll Isopaque gradient centrifugation method [4] The interphase cells were collected and cell preparations were made for MGG staining and for autoradiography as previously described On the average, 95 7% of the cells were lymphocytes and 1 2% were immature myeloid cells The percentage of cells that formed rosettes (E RFC) with sheep red cells (SRC) was determined as described elsewhere [20] except that in the present study the cells were suspended in medium containing 20% CS instead of in serum free medium

Separation of E RFC To separate out the E RFC, a velocity sedimentation method was tried and found satisfactory 4 ml of the rosette suspension (8×10^6 lymphocytes, 0.5% SRC) was layered on top of 1 ml CS and centrifuged for 10 min at 10 g The supernatant on top of the serum and the pellet that was gen

tly resuspended in 2 ml of medium containing 20% CS were analyzed for E-RFC and non E RFC. Cytocentrifuge preparations for MGG staining and for autoradiography were made both directly from the two fractions, and after haemolysis of SRC with ammonium chloride and the subsequent concentration of lymphocytes. Rosette-containing preparations were fixed with benzidine reagent [3] for better visualization of SRC. Autoradiographs were prepared and stained as previously described.

Results

Labelled cells in cord blood. The percentages of labelled cells in the cord blood of the 12 healthy newborn infants are given in table I. In all cases the preponderance of labelled cells were LEC or LMC or both but their percentages varied greatly. The percentage of LLC on the other hand was uniformly low.

Labelled cells in the blood of healthy newborns. The percentages of LLC and LMC in the blood of healthy infants between the ages of 0 and 12 days are shown in figure 1. During the first 2 days only about 0.1% of the mononuclear cells were LLC. At 3 days the number of LLC started to rise and by 4–7 days after birth their number had increased tenfold as compared to the numbers present immediately after birth. Thereafter the percentage of LLC gradually decreased.

The numbers of LMC were variable, and the scatter of their percentages was wider than that for LLC. During the first 3 days after birth the majority of the cells labelled with ^{3}H-TdR were immature myeloid cells. By the 12th day the number of LMC had gradually decreased from about 0.5 to 0.1%.

Labelled erythroid cells were present in most of the samples taken from infants under the age of 12 h (highest value 1.3%). In the blood of

Table I. Labelled cells expressed as a geometric mean percentage of the mononuclear cells, in 12 samples of cord blood

Labelled cells	Geometric mean percentage	Range
Lymphoid	0.08	0.01–0.35
Myeloid	0.74	0.17–4.03
Erythroid	0.69	0.06–10.3
Total	1.99	0.29–11.8

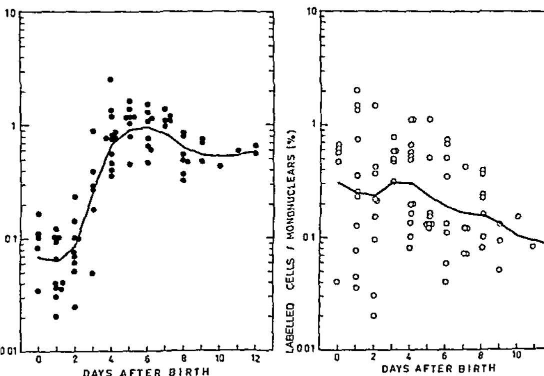


Fig 1 Labelled lymphoid cells (a) and labelled myeloid cells (b) as percentages of mononuclear cells in the neonatal period Moving geometric means in the order of 3 (weighed 1, 2 1) are connected with a line

infants older than 12 h, LEC were seen only occasionally, and then in percentages lower than 0 2%

In conventional differential counts the percentage of lymphocytes increased from 31% at the age of 1 day to 58% at the age of 7 days This increase was mainly the result of a decrease in the absolute number of neutrophils, while the absolute number of lymphocytes remained essentially the same throughout the follow-up period

No correlation could be found between the number of LLC and LMC and the duration of gestation, the sex, or the birth weight

Characterization of proliferating lymphoblasts After purification with iron and separation by Ficoll-Isopaque gradient centrifugation, 74 5 ± 11 5% (SD) of the lymphocytes from 5- to 7-day-old infants formed rosettes with SRC This percentage is somewhat lower than that for healthy adults studied in the same laboratory (78 5 ± 5 3) [20]

After 10 *g* velocity sedimentation, an average of 95% of the cells in



Fig 2 A labelled (left) and a non labelled (right) rosette forming lymphoid cell in a 6-day-old infant

the bottom fraction and 40% in the supernatant were E-RFC. In the benzidine-stained cytocentrifuge preparations from the bottom fractions, rosettes were well preserved (fig 2), and more than 90% of the labelled cells were E RFC Most of the non-rosette-forming cells in these preparations were LMC In the benzidine-stained preparations from the supernatant fractions, the large number of SRC obstructed visualization of rosette formation Quantitative counts of the labelled cells were done on the haemolyzed preparations As shown in table II, the bottom fractions were enriched with LLC as compared with the non fractionated suspensions On the other hand, most supernatant fractions were enriched with LMC. The ratio of LLC to LMC was, on the average, 8 1 in the bottom fractions, and 1 2 1 in the supernatant fractions

Discussion

Isotope incorporation methods employing labelled DNA precursors have been widely used to study the proliferation of blood leucocytes In studies on adults, conventional sedimentation procedures for concentrating leucocytes in buffy coat of venous blood are easily carried out and were used in this investigation to study cell proliferation in cord blood

Table II Percentages of labelled cells before and after separation of E RFC with 10 *g* velocity sedimentation

Case	Age days	Non fractionated total	LLC	LMC	Bottom fraction total	LLC	LMC	Supernatant fraction total	LLC	LMC
1	5	1.80	1.67	0.13	1.72	1.67	0.05	0.26	0.02	0.24
2	5	1.13	0.88	0.24	1.24	1.16	0.08	0.67	0.27	0.40
3	5	1.72	1.41	0.31	1.59	1.07	0.52	0.72	0.36	0.36
4	5	2.35	2.09	0.26	2.17	2.13	0.04	0.72	0.57	0.15
5	5	3.02	2.70	0.32	2.22	2.01	0.21	1.05	0.78	0.24
6	5	1.71	1.24	0.47	2.02	1.72	0.30	0.95	0.56	0.39
7	6	1.92	1.33	0.53	2.11	1.77	0.31	1.27	0.40	0.87
8	7	0.87	0.83	0.04	1.01	0.97	0.04	0.35	0.34	0.01
$\bar{X}$		1.82	1.52	0.29	1.76	1.56	0.19	0.75	0.41	0.33
SD		0.67	0.63	0.16	0.45	0.44	0.17	0.34	0.23	0.25

For newborn infants, however, a method calling for smaller quantities of blood is more applicable, and a technique that involved 50 μl samples of capillary blood, from which mononuclear cells were separated by Ficoll-Isopaque gradient centrifugation, was found to be satisfactory. The results achieved with this technique were comparable to those obtained with sedimentation of venous blood [11].

DNA synthesizing cells in human blood can be grouped into three main categories: erythroid, myeloid, and lymphoid cells [10]. In healthy adults, about 0.1% of the mononuclear cells are LLC [13]. LMC are also regularly present but in about ten times lower numbers, whereas LEC are virtually never found in blood of healthy adults [21]. In cord blood and in blood of early newborns, immature erythroid cells constitute a substantial part of the circulating mononuclear cells. The absolute and percentage counts of nucleated red blood cells are highest at birth, within 18 h their numbers decrease abruptly [14, 16, 18]. Immature myeloid cells are regularly present during the first days of life [8, 14, 23]. EFRATI *et al* [8] reported that myelocytes and metamyelocytes make up 2.5% of the leucocytes in cord blood and XANTHOU [23] recorded the presence of up to 2,000 metamyelocytes/μl of blood in healthy infants during the first 3 days of life. Both immature erythroid and myeloid cells have virtually disappeared by the end of the first week. During the first week of life neutrophils are known to diminish in number and the absolute white blood cell counts decrease as well, the

absolute lymphocyte counts however vary little [1, 14, 23]. In the present study, LLC were numerous in cord blood, but their number decreased abruptly within the first 12 h after birth. Large numbers of LMC were present until the 4th day of life, when their numbers gradually started to decrease. Thus, the incidence and distribution of DNA synthesizing erythroid and myeloid cells during the neonatal period parallels the numerical pattern of these cells as revealed in conventional differential counts.

Lymphoblasts increase in number in viral diseases, after immunizations, and in various other clinical conditions [7, 12, 13, 22]. In sheep, lymphoblasts are present in the intestinal lymph in vastly increased numbers immediately after birth [15]. In a few studies on lymphoid cells in newborns, higher labelling indices than those normally characteristic of adult blood have been reported. WINTER *et al.* [19] and FAULK *et al.* [9] in their studies of cord blood, reported ^{3}H-TdR labelling indices for lymphocytes that are about equal to the total labelling index in the present study. However, nearly all the labelled cells in cord blood in the present study were identified as either erythroid or myeloid cells. In the study of FAULK *et al.* [9] most of the cells seemed to be weakly labelled, and the morphology of the 'high labelling cell' would appear to resemble more that of a cell in the myeloid than in the lymphoid series. ANDERSEN and ANDERSEN [2] reported an increase in the count of ^{3}H-TdR labelled cells from day 3 to 6 after birth in healthy newborns which agrees with the present results. Contrary to the present findings, however, they observed an initial decrease in labelled lymphoid cells between days 1 and 3, but the possibility that early myeloid cells were included in their count was not ruled out.

In the present study a greater than tenfold increase in the percentages of LLC occurred 4–7 days after birth in healthy infants, an increase that resembles the previously reported response of the organism to various immunizations. Whether the LLC that were detected in the blood after birth belonged to the T or the B lymphocyte population was determined by making use of the capability of T lymphocytes to bind SRC on their surface [5, 6]. The proportion of T cells in the blood of the 5- to 7-day-old infants in this study did not differ significantly from that in adults.

The 10 *g* centrifugation method used here to segregate rosettes separates particles according to their size rather than their density. This method was used instead of the 1 *g* velocity sedimentation method previously employed [20], because it is less complicated and faster. T RFC

Acta haemat 53 138–144 (1975)

Intracellular Potential in Normal and Leukemic Lymphocyte

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Abstract Lymphocytes obtained from fresh blood of patients suffering from chronic lymphatic leukemia have a lower intracellular potential than normal lymphocytes Compared to normal lymphocytes, the intracellular potential in leukemic lymphocytes behaves differently in environments with a changing concen tration of K and Cl ions

Key Words
Electrobiology
Intracellular lymphocyte potential
Lymphatic leukemia

Blood cells show only the 'membrane' or 'intracellular' potential whic is measured by a difference of potentials on both sides of the cell membrane Up to the present, the behavior of the intracellular potential in norma leukocytes [1], erythrocytes [3] and lymphocytes from the culture *in vitro* [5 and those obtained from the fresh peripheral blood [4] has been investigated

In the present investigations, we have compared electrobiologica characteristics in fresh normal lypmhocytes and in the lymphocytes of bloo from patients with chronic lymphatic leukemia

Materials and Methods

In the peripheral blood of normal subjects, lymphocytes constituted 20–40% of nucle ated blood cells, and in the peripheral blood obtained from patients suffering from chronic lymphatic leukemia lymphocytic cells comprised 65–95%

We have studied lymphocytes obtained from the peripheral blood of 20 norma persons 20–60 years old (more than 200 measurements) as well as from the periphera blood of 10 patients 30–65 years old suffering from chronic lymphatic leukemia (more than 150 measurements) The patients had not been given corticoids and cytostatics a week prior to the experiment

Table I Composition of nutritive solutions, in mM[1]

Nutritive solution	Changes of concentration	Na^+	Cl^-	K^+
Standard	–	137.2	146.17	2.68
Reduction in Cl	2 fold	137.2	73.1[1]	2.68
	27 fold	137.2	5.9[1]	2.68
Changes in K^+	10-fold increase	115.35	147.89	26.7
	10-fold decrease	137.2	143.7	0.27

The other ion concentrations as in Tyrode's solution

[1] In the nutritive solutions, sodium sulfate or potassium sulfate were substituted for sodium chloride or potassium chloride

The determinations were carried out on a suspension of lymphocytes from fresh blood obtained from the cubital vein. The blood was immediately mixed with 3.8% sodium citrate (9:1). Glass vessels, drains and plastic vessels were coated with several layers of silicon (Siliclad, Clay Adams). The Jago method [2] was used to separate cells, which uses differences in the density of lymphocytes. The suspension contained on an average 95% *lymphocytes, 4% monocytes and 1% other nucleated cells. The suspension was made up* to 10^4 lymphocytes/mm³ with plasma from the investigated persons.

Immediately before the determinations, 0.2 ml of the lymphocyte suspension was taken into the mixture of 0.4 ml Tyrode's solution and 0.4 ml 1% agarose solution. The final concentration of lymphocytes was 2×10^4/mm³. The whole mixture was then placed into a glass container in which a constant temperature was maintained by means of an Ultra thermostat. When agarose congealed, the whole suspension was covered with Tyrode's solution. The determinations were made at 23°C.

Electrobiological determinations were made by means of a Pyrex glass microelectrode (external tip diameter less than 0.5 μm and 20–50 MΩ resistance) filled with 3 M KCl. The microelectrodes were made on a horizontal automatic puller constructed by the Medical Electronic and Technical Laboratory of the Experimental and Clinical Medical Centre of the Polish Academy of Science. The tip potential of the electrode did not exceed 10 mV.

The electrode was placed in a micromanipulator and, under microscopic control, introduced into a lymphocyte immobilized by congealed agarose. The potentials recorded by electrode were transmitted to a direct current amplifier and displayed on the screen of an oscilloscope. They were recorded with a Recordine camera. The neutral Ag–AgCl electrode was placed in the lymphocyte suspension (Fig. 1). A typical 'transmembrane' or intracellular potential recorded from a single lymphocytic cell is shown in figure 2. The magnitude of the transmembrane potentials has been constant and has remained on an unchanged level until the electrode was taken back from the cell.

The determinations were carried out in agarose gels made up with Tyrode's solution, the composition of which being changed as required. A constant osmolarity of different

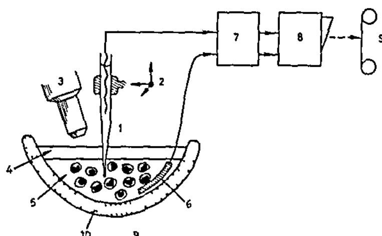


Fig 1 Method of electrobiological measurements 1 = Microelectrode, 2 = micromanipulator, 3 = optical system, 4 = layer of Tyrode's solution, 5 = lymphocytes in the net of congealed agarose, 6 = zero electrode Ag-AgCl, 7 = DC amplifier, 8 = oscilloscope, 9 = film camera, 10 = container maintaining a constant temperature



Fig 2 Typical reading of electrobiological phenomena from the inside of lymphocytes On the left calibration = 5 mV, A = impalement of the microelectrode into a cell, B = moment of the electrode removal from a lymphocyte Observation time 2 min

solutions was achieved by adding saccharose Table 1 shows the composition of the solutions The lymphocyte solution was incubated in a nutritive fluid of a determined ion concentration, constantly oxygenized for 30 min at 23°C

The statistical significance of differences between the results obtained from different groups of experiments was evaluated by means of a variable determined by the formula

$$U = \frac{\bar{X} - \bar{Y}}{\sqrt{n_1 s_1^2 + n_2 s_2^2}} \sqrt{\frac{n_1 n_2}{n_1 + n_2}(n_1 + n_2 - 2)}$$

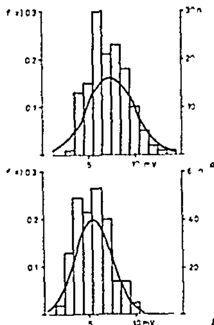


Fig. 3. Histograms of the intracellular potential of lymphocytes. *a* Lymphocytes of normal subjects. Continuous line = curve of a normal distribution of Gauss N (7.42; 2.54). *b* Lymphocytes of patients suffering from chronic lymphatic leukemia. Continuous line = curve of a normal distribution of Gauss N (5.62; 2.04). Left vertical axis = theoretical frequency of normal distribution f(x). Right vertical axis = number of measurements (n). Horizontal axis = intracellular potential (mV).

where $\bar{X}$, $\bar{Y}$ = mean values determined from samples; n_1, n_2 = number of measurements included in the samples; s_1, s_2 = standard deviations determined from the samples.

The variable t has a Student t distribution of (n_1+n_2-2) degrees of freedom. A disagreement between the normal distribution and empirical one was tested by means of χ^2 test.

Results

Intracellular potential. The mean value of the intracellular potential of lymphocytes from the peripheral blood is -7.4 ± 0.2 mV. The mean value of the intracellular potential of lymphocytes from the blood obtained from patients suffering from leukemia is -5.6 ± 0.1 mV. This is a statistically significant difference. The intracellular potentials of all measurements in

Table II The intracellular potentials in relation to potassium ion concentration

Extracellular concentration of potassium ions, mM/l		Normal lymphocytes mV	Leukemic lymphocytes mV
10 times higher	26 8	5 8±0 4	5 4±0 4
Normal	2 68	7 4±0 2	5 6±0 1
10 times lower	0 268	9 5±0 7	9 1±0 8

Table III Intracellular potentials of normal and leukemic lymphocytes in the environment with a changing concentration of potassium ions

Extracellular concentration of K^+ ions mM/l	Normal lymphocytes %	Leukemic lymphocytes, %
10 times higher	78 4	96 4
Normal	100 0	100 0
10 times lower	128 4	162 5

The value of the intracellular potential of lymphocytes in normal Tyrode s solution has been taken as 100%

normal and leukemic lymphocytes are presented in figure 3 The use of the $\chi 2$ test indicates that the probability of accordance of the observed distribution with the distribution of Gauss is

$$p(\chi^2 \geq 5\ 86) > 0\ 30 \text{ and } p(\chi^2 \geq 6\ 72) \sim 0\ 20$$

for normal and leukemic lymphocytes, respectively

Influence of concentration changes of K^+, Cl and Na^+ ions upon the intracellular potential of lymphocytes The value of the lymphocyte intracellular potential depends on the potassium ion concentration in the extracellular environment and is a linear function of a logarithm of this concentration The intracellular potential of lymphocytes from leukemic blood, however, does not show a linear dependence on the logarithm of potassium ion concentration in the nutritive fluid The results are summarized in table II

Differences of the intracellular potential between normal and leukemic lymphocytes are still more evident, when we assume conventionally in both

Table IV Intracellular potentials of normal and leukemic lymphocytes in Tyrode's solution and in the solution with low concentration of Cl ions

Solution	Normal lymphocytes, mV	Leukemic lymphocytes, mV
Normal Tyrode's solution	7.4±0.2	5.6±0.1
Solution with lowered Cl concentration	5.4±0.2	5.5±0.4

groups the value of the transmembrane potential in the medium of Tyrode's solution with a normal ionic composition as 100% (table III) Compared to a linear dependence in normal lymphocytes, in leukemic lymphocytes the transmembrane potential increases rapidly when the potassium concentration is low In the medium containing potassium ions in 10 times higher concentrations the potential practically does not change

Table IV represents the behavior of the transmembrane potential in normal and leukemic lymphocytes depending on the chlorine ion concentration in the outer environment A decrease from 146 17 to 5 9 mM causes a decrease in the intracellular potential to about -5 5 mV Compared to normal lymphocytes, in leukemic lymphocytes the removal of chlorine ions from the medium does practically not change the intracellular potential Furthermore a gradual change of the natrium ion concentration in the nutritive solution does not influence significantly the intracellular potentials of both normal and leukemic lymphocytes

Discussion

The investigations indicate that the inside of lymphocytes has a negative potential in relation to the external medium (-7 6 mV) Changes of the intracellular potential depend mainly on the external potassium concentration The second ion influencing the intracellular potential is chlorine

The intracellular potential depends upon many factors morphological and functional states of the cell membrane, ion concentration on both of its sides as well as on biochemical processes taking place in the cell inside Lymphocytes of leukemic blood possess quite different electrobiological properties which might result from different mechanisms conditioning or controlling ion transport through the cell membrane

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Table IV represents the behavior of the transmembrane potential in normal and leukemic lymphocytes depending on the chlorine ion concentration in the outer environment. A decrease from 146.17 to 5.9 mM causes a decrease in the intracellular potential to about −5.5 mV. Compared to normal lymphocytes, in leukemic lymphocytes the removal of chlorine ions from the medium does practically not change the intracellular potential. Furthermore a gradual change of the natrium ion concentration in the nutritive solution does not influence significantly the intracellular potentials of both normal and leukemic lymphocytes.

Discussion

The investigations indicate that the inside of lymphocytes has a negative potential in relation to the external medium (−7.6 mV). Changes of the intracellular potential depend mainly on the external potassium concentration. The second ion influencing the intracellular potential is chlorine.

The intracellular potential depends upon many factors: morphological and functional states of the cell membrane, ion concentration on both of its sides as well as on biochemical processes taking place in the cell inside. Lymphocytes of leukemic blood possess quite different electrobiological properties, which might result from different mechanisms conditioning or controlling ion transport through the cell membrane.

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Acta haemat 53 145-151 (1975)

Localization of Leukocyte Alkaline Phosphatase in Human Neutrophils[1]

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Abstract The enzymes of blood and marrow neutrophils can be used to aid in the determination of the cell's maturity The enzyme marker which signals the production of secondary granules leukocyte alkaline phosphatase (LAP) was found to be inactivated by aldehyde fixation but minimal fixation of cells gave adequate marphological preservation and good enzyme activity Notably, LAP activity was found to be present in neutrophils from the marrow myelocyte stage to the blood neutrophil stage and high levels were found in the synthetic organelles of some circulating mature neutrophils. These observations at the electron microscopic level confirm the classical light microscopic histochemical findings.

Key Words
Cytochemistry
Electron microscopy
Golgi activity
Human neutrophils
Leukocyte alkaline phosphatase

Leukocyte alkaline phosphatase (LAP) is a potential marker for defining the maturation sequence of neutrophils in human marrow and peripheral blood At the light microscopic level using azo dye techniques [7], LAP activity is first observed at the myelocyte stage and this activity increases through the circulating mature neutrophil stage [1] Other leukocyte enzymes, notably acid phosphatases and peroxidases, behave in the opposite manner, i e, activity is greatest in the promyelocytes and decreases with maturation This pattern is similar to the enzyme fluctuations seen in the rabbit heterophil series

In the rabbit, enzyme heterogeneity has been correlated with granule

[1] Supported in part by grants ACS IN 21M and NIH CA 05156-13

heterogeneity [4] Acid hydrolytic enzymes have been reported to be restricted to the primary granules synthesized at the incipient promyelocyte stage whereas other enzymes, specifically LAP, are believed to be produced and packaged in the secondary granules at the myelocyte stage

On morphologic criteria alone, ACKERMAN [2] and SCOTT and HORN [10] have proposed at least two granule populations in normal human mature neutrophils The work of BAINTON *et al* [5] supported the distinctness of enzyme content in two granule types in human neutrophils at the electron microscopic level However, LAP activity was confined to the myelocyte stage only, where reaction deposits appeared in the Golgi cisternae and within a few scattered granules NAKATSUI [8] demonstrated LAP activity rimming some granules and vacuoles in unfixed neutrophils from peripheral blood In the metamyelocyte and mature circulating neutrophil, this enzyme has not previously been demonstrated in synthetic organelles or to any great extent in the granules

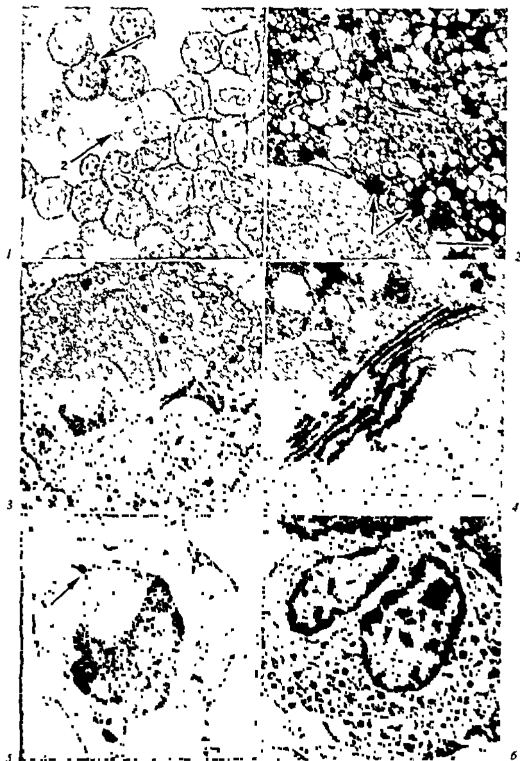
Materials and Methods

Bone marrow was aspirated into a heparinized syringe using standard technique from the posterior iliac crest of normal volunteers and patients with nonhematologic neoplasms The latter marrows were obtained for evaluation of marrow status prior to chemotherapy The marrow was expressed onto a small watch glass and the cellular particles removed with a Pasteur pipet, smeared on polyester plastic slides (Mylar) and force air dried Peripheral blood and marrow blood buffy coats were prepared using microhematocrit tubes After centrifugation each tube was scored between the sedimented red cell and leukocyte layers The tube was then snapped and the leukocyte layer expressed with a little plasma onto a plastic slide and smeared as above Dried smears were fixed for 5 min in Coplin jars filled with various concentrations (5–0.5%) of cold glutaraldehyde (25% stock Polysciences) in 0 1 M cacodylate buffer pH 7 4 After fixation smears were washed for a minimum of 5 min in tap water or 0 1 M cacodylate buffer, allowed to dry and incubated in the reaction medium for 1 h in a 37 °C water bath The incubation medium was a modification by WETZEL *et al* [11] of the GOMORI procedure [6] for LAP The substrate used was β glycerophosphate grade 1 (Sigma) Controls consisted of smeared cells incubated in media without the substrate After incubation the slides were tap water rinsed flooded with 2% lead nitrate for 5 min, rinsed and briefly covered with dilute ammonium sulfide to visualize the cellular areas more easily Slides were then processed for electron microscopy by the usual method of postfixation for 1 h in 1% osmium tetroxide alcohol dehydration brief propylene oxide wash, and infiltration with a 1 1 mixture of propylene oxide and Araldite 502 resin Beem capsules were partially filled with Araldite and inverted over the cellular areas of the slide

Polymerization took approximately 48 h in a 60 °C incubator. The capsules were then snapped off the plastic slides leaving the cells embedded in the resin. The top of the block containing the monolayer of cells was then trimmed and thick and thin sections were cut with a diamond knife. Micrographs were taken without poststaining on a Hitachi 7S electron microscope.

Results

With minimal fixation of cells at a low concentration of glutaraldehyde (0.5%), LAP was observed in the granules and synthetic apparatus of the maturing marrow neutrophils and in the circulating mature neutrophils. Preservation of morphologic detail was improved by longer fixation (or increased concentrations of glutaraldehyde, 1.0–5%) but enzyme activity decreased concomitantly. At the light microscopic level, LAP activity as determined by our modified Gomori technique correlated well qualitatively and quantitatively with the LAP activity in cells reacted by Kaplow's azo dye (fig. 1) and in fact proved to be more sensitive in picking up activity in the more immature granulocytes. For instance, in cell counts of a thousand cells, the percentages of cells exhibiting enzyme activity were: myelocytes, 27%, metamyelocytes, 74%, band forms, 91%, and polymorphonuclear leukocytes (PMNs), 94%. With our modified Gomori method the percentages positive were 34, 81, 92, and 95%, respectively. For every individual the intensity and proportion of neutrophil staining was dependent upon a number of factors, such as the patients' inflammatory and hormonal status. However, even in patients with a severe leukemoid reaction, an occasional mature neutrophil exhibited minimal or no reactivity as shown in fig. 6. Dense deposits of the reaction product were localized in the perinuclear cisternae (fig. 3), the Golgi complex (fig. 3, 4) and the granules (fig. 2) of the marrow and peripheral neutrophils. The Golgi complex when reactive was totally involved, and exhibited no evidence of polarization, as has been suggested for the Golgi complexes of rabbit and human neutrophils [3]. Reaction deposits were also present in phagolysosomes. Figure 5 demonstrates a reactive granule emptying its contents into a phagosome forming a secondary lysosome in a marrow neutrophil from a normal subject. Extraneous background precipitate was commonly observed, especially over the heterochromatin of the nucleus, and frequently throughout the cytoplasm. The omission of substrate from the incubation medium resulted in no activity.


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Discussion

The inability to demonstrate LAP at the electron microscopic level has been attributed to a structural linked latency of the enzyme [5]. This latency probably determines the minimum LAP activity demonstrable by lead salt techniques but the sensitivity of the demonstration of any LAP above this minimum is dependent upon the concentration of glutaraldehyde employed in cell fixation. Glutaraldehyde, which is the fixative of choice for most electron microscopic histochemical studies [9], inhibits the reactivity of LAP. Various concentrations of other fixatives (paraformaldehyde, acrolein, acetone, methacrolein, glyoxal) used alone, in combination, and in sequence with one another have been assessed for their ability to preserve enzyme activity. Leukocytes fixed in 60% acetone exhibited the most extensive enzyme reactivity but cell preservation at the electron microscopic level was inadequate. In drastically reduced concentrations glutaraldehyde gave the best combination of enzyme reactivity and morphological preservation. Some disruption and lysis of organelles was expected due to the incubation of the minimally fixed cells for 1 h in a medium pH 9.0–9.3.

The discrete containment of enzyme reactivity by the saccules of the Golgi membrane, the perinuclear cisternae and membranes of some granules argues that the reaction deposits are true sites of enzyme activity rather than artifactual staining. The partial reactivity of perinuclear cis-

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Fig. 1. Light micrograph of Araldite thick section of peripheral human neutrophils reacted for LAP and counterstained with methylene blue. Arrow 1 points to a neutrophil reactive for LAP; neutrophil at arrow 2 exhibits little or no reactivity. × 900

Fig. 2. Electron micrograph of a portion of a mature reactive neutrophil from blood with artifactual cytoplasmic staining. Note positive (arrows) and negative granules; the latter apparently correspond to LAP-deficient primary granules. No heavy metal poststaining in figures 2–6 and all lines are one micron. × 14,000

Fig. 3. Mature blood neutrophil reactive for LAP. Note reactive Golgi complex, nuclear cisterna, and granules. Mitochondrion at m. × 7000

Fig. 4. Golgi complex with well localized LAP activity in a peripheral neutrophil. × 2[illegible]000

Fig. 5. Secondary lysosome in a marrow neutrophil (myelocyte stage). Note confluence with a reactive granule (arrow). × 12,000

Fig. 6. Mature blood neutrophil with membrane fragmentation exhibiting no LAP reactivity. × [illegible]

ternae may be due in some cases to the plane of cut of the section or to the fixation of cells at the beginning or end of LAP synthesis The synthesis of only one product at a time through the cell organelles has never been conclusively demonstrated

Our results could not definitely characterize the reactive granules as specific granules Besides enzymology, the other criterion for distinguishing specific from azurophilic granules is their morphology which is still disputed among electron microscopists In addition, the morphology of these granules changes with cell maturation and is susceptible to preparative artifaction Relevant information on the heterogeneity of human PMN granules must come from further enzymatic or functional data

In the majority of preparations reaction deposits were seen at the cell periphery in an extracellular location (fig 3) The extraneous precipitate could be due to the fact that (1) the lead solution which couples with the enzyme product to form an insoluble deposit is not added until the end of the incubation period during which diffusion of enzyme product could occur, or (2) the extractive properties of the solvents used in subsequent processing could result in diffuse precipitate

With the variation in fixation procedure, LAP reaction was observed in the synthetic apparatus of neutrophils from the peripheral blood This finding indicates that the circulating neutrophil is not the synthetically inactive cell as had been generally hypothesized

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Acta haemat 53 152–157 (1975)

Erythrocyte Glucose-6-Phosphate Dehydrogenase in Tehran

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Abstract Eryhrocyte glucose 6-phosphate dehydrogenase (G 6-PD) of 370 randomly selected patients referred to the pediatric service of Pahlavi Medical Center, Tehran, was studied A deficiency was found in 42 7% Erythrocyte G 6 PD of the 58 subjects with normal level of the enzyme and 31 patients with deficiency had the electrophoretic mobility of variant B Fast electrophoretic band (105–110% of normal) was present in 8 and slow mowing band (85–93% of normal) in 7 cases.

Key Words
Glucose 6-phosphate dehydrogenase
G 6-PD deficiency in Tehran
G 6-PD variants

Erythrocyte glucose-6-phosphate dehydrogenase (G-6-PD) deficiency is a common enzymatic abnormality in Iran [8] resulting in various clinical manifestations This might well be due to the different variants in the deficient population in this country [15] We present here the results of the study performed on the frequency of the G-6-PD in Tehran and electrophoretic mobility of the enzyme in the deficient and non-deficient population

Material and Methods

Erythrocyte G 6-PD of 370 randomly selected patients referred to the pediatric service of Pahlavi Medical Center in Tehran was studied

Enzyme quantitation was performed by the method recommended by the WHO at 25 °C [3] and by the ultraviolet spectrophotometric method using kit manufactured by Boehringer GmbH (Mannheim, FRG)

Electrophoresis was performed on cellulose acetate (Celagam Shandon) using Tris EDTA borate buffer, pH 9 8 [28] and on starch gel (starch hydrolyzed Con naught) using the electrophoretic technique recommended by the WHO [29] Hori

zontal starch gel electrophoresis was performed using Tris as well as phosphate buffer 1/20 dilution of the hemolysate was used for electrophoresis.

Results

In 370 patients studied, 158 (42.7%) were G-6-PD deficient. The enzyme activity varied from 0 to 82.5% of normal in these patients. The rest of the subjects had activities of 89.55–224.8% of normal. The main clinical findings in deficient subjects are shown in table I. 33 patients (20.9%) presented hemolytic anemia following fava bean ingestion. Nonspherocytic hemolytic anemia was seen in 14 of them (8.9%). Other hematological diseases were the clinical manifestations of 12.6% of the deficient group. 57.6% of the deficient subjects were asymptomatic. The level of G-6-PD was significantly lower in hemolysis following fava bean ingestion and in nonspherocytic hemolytic anemia (table I).

Among 24 relatives of the G-6-PD deficients, 74.7% had low enzyme activity. All the patients with hemolytic anemia following fava bean ingestion were G-6-PD deficient, while none of the patients with thalassemia (24 cases) and iron deficiency anemia (32 cases) showed any deficiency.

Starch gel and cellulose acetate electrophoresis of 65 subjects with normal G-6-PD level and of 52 patients with G-6-PD deficiency were stud-

Table I. Clinical manifestations in deficient subjects

	Male	Female	Total	%	Erythrocyte G-6-PD activity %	p
Hemolytic anemia following fava bean ingestion	23	10	33	20.9	26.4 ± 25.8 (0–48.6)	< 0.001
Nonspherocytic hemolytic anemia	9	5	14	8.9	2.7 ± 5.8 (0–19.4)	< 0.001
Other hematological diseases	16	4	20	12.6	41.3 ± 28.3 (7.9–80.6)	
Asymptomatic	70	21	91	57.6	41.9 ± 24.5 (0–82.5)	0.05 < p < 0.1
Total	118	43	158	100		

Table II Electrophoretic mobility in G-6-PD normal and deficient subjects

		G-6-PD variant		
		B	fast	slow
Deficient	male	18	4	4
	female	13	–	–
	total	31	4	4
Non-deficient	male	32	4	3
	female	26	–	–
	total	58	4	3

Table III Erythrocyte G-6-PD activity and electrophoretic mobility in G-6-PD deficient subjects

G-6-PD variant	Number studied	Erythrocyte G-6-PD activity, %	Electrophoretic mobility, %
B	31	22.6±19.9 (0–68.7)	100
Fast	4	25.8±29.9 (0–54.3)	105–110
Slow	4	20.8±25.8 (0–53.1)	85–93

ied (table II) 31 patients with low G-6-PD level (18 male and 13 female) had electrophoretic mobility of variant B A faster electrophoretic band was present in 4 patients with low G-6-PD level (all male) The 4 other deficient male subjects showed the slow moving band 13 patients with very low level of enzyme (8 male and 5 female) did not show any electrophoretic band

58 subjects with normal G-6-PD level had an electrophoretic pattern compatible with variant B A fast moving electrophoretic band was found in 4 and a slow moving one in 3 of the subjects with normal enzyme activity

Table III shows the erythrocyte G-6-PD activity and electrophoretic

mobility in deficient subjects The faster band was 105–110% of normal, and the slow moving band was 85–93% of normal variant B

Discussion

G-6-PD deficiency is found in 2–35% of the world area [20] in a wide distribution [1, 2 8, 10–14, 16 20–23, 26] The high incidence in the Mediterranean area is thought to be related to genetic polymorphism in which G 6 PD deficiency protects against fatal falciparum malaria [11, 23] However, this protection was not found in some areas [9] GdA /GdB' females are most protected [6] Glutathione disulfide in G-6 PD deficient red blood cells inhibits protein synthesis by the intracellular parasite [19] The percentage of G-6-PD deficiency in this study was 42 7% The percentage was almost the same in the symptom free control group, and this is higher than that reported previously by BOWMAN and WALKER [8] in Iran This could partly be due to the quantitative estimation of the enzyme which detects partially deficient subjects as well

A different clinical manifestation is seen in G-6-PD deficient subjects due to the level and biochemical characteristics of the enzyme [15, 31] Hemolysis following fava bean ingestion is common in Mediterranean type B variety [18], however not all the patients with G-6-PD deficiency have clinical favism [7] The presence of G-6-PD deficiency is necessary for the occurrence of favism but not sufficient A serum deficiency is suggested to be a cause of favism [20] An autosomal gene for acid phosphatase may be another factor [7] Fava beans are the major source of large amounts of free β-(3 4 dihydroxyphenyl)-L alanine (L-dopa) and hemolysis is dependent upon the rate of production of dopa quinone production from L-dopa [5] therefore increased tyrosinase activity may be the addition factor [4] It was very interesting to note that some of our patients had ingested fava bean previously without developing any symptoms This sudden reaction is not unusual in favism [15] Hemolytic crisis of favism is seen in heterozygotes as well As only enzymopenic red cells are selectively lysed the clinical signs may be minimal [27]

More than 70 G-6-PD variants have been discovered since 1967 [30] The 2 major molecular forms of G-6-PD within red cells are types A' and B' The molecular difference appears to be a single amino acid

substitution, asparagin in B+ for aspartic acid in A+ [15] Approximately 18% of American Negro males have A+ [23, 24] and almost all Caucasians have B+ [25] In this study, most of the cases showed electrophoretic mobility of variant B

The fast electrophoretic band (110% of normal) which was present in 3 patients with enzyme deficiency and in 2 individuals with normal level of enzyme, is compatible with variant A [17] The enzymes of fast and slow electrophoretic mobility are the variants which might be different to what has already been known We are studying this matter further

Acknowledgements The author is grateful to Prof N GUITI (Head of the Department of Experimental Medicine and Pharmacology) and Dr S RAHBAR (Head of the Department of Applied Molecular Biology) for their helpful advice to Miss M FARSHIAN for her technical assistance and to Mrs N SAMADI for her aid in preparing the manuscript

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Acta haemat 53 158-164 (1975)

Extract of *Fagara zanthoxyloides* Root in Sickle Cell Anaemia

Toxicology and Preliminary Clinical Trials

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Abstract Following the demonstration of the ability of aqueous extracts of *Fagara zanthoxyloides* root to reverse sickling, toxicological studies in duck and chick embryos as well as in mice have shown these extracts to be non toxic Preliminary clinical trials demonstrated a significant diminution in the incidence of painful episodes in *Fagara* treated individuals

Key Words
Fagara root extract
Sickle cell anaemia
Sickling prevention

The detailed knowledge of sickle cell anaemia now available at the molecular level has stimulated various attempts to improve the management of this disease The major efforts are now directed to preventing the homozygous inheritance of the disease by genetic counselling [16], prenatal detection and selective abortion [8] as well as by interfering with the process of sickling, with antisickling agents Urea [12] and potassium cyanate [2, 4] are perhaps the best known of the antisickling agents which are being currently investigated They respectively interfere with hydrophobic bonds which contribute to the gelation process by binding to the N-terminal residue of globin chains thus changing the oxygen affinity of the haemoglobin in the process However, a group of steroids, in particular testosterone and progesterone, have also *in vitro* and *in vivo* efficacy as antisickling agents [1, 5–7] Similarly, the observation that aqueous extracts of *Fagara zanthoxyloides* root preserved the red colour of blood in blood agar culture plates [3] had led to the description of an antisickling effect in that root extract [14] Further investigation has confirmed this

effect and a pure substance with a potent antisickling activity has been characterized from the root and synthesized [15] in our laboratories

Since antimicrobial activity against oral microorganisms was also demonstrated with this root extract [3] its potential in the prevention of sickle cell crises (often precipitated by upper respiratory infections) is clear

This report describes the findings of toxicological examination of the aqueous extract of this chewing stick and the preliminary clinical assessment of it on sickle cell disease patients

Materials and Methods

Preparation of the Extract

The aqueous extract was prepared by soaking the chopped roots of the 'Orin ata' chewing sticks – authenticated as earlier specified [3, 14] – in equal weight of distilled water. The preparation was left in the refrigerator at 4 °C and decanted after 2 days. This produced an extract containing 1 mg of water-soluble extractive/ml. For the toxicological studies requiring concentrated extract, the powdered root was exhausted with water in a soxhlet extractor. The water-soluble extract was then concentrated *in vacuo* to the required strengths as necessary

Embryo Toxicology Studies

Method 1. The primary cell cultures were prepared by adopting the method of Mirchamsy *et al* [11]. Duck and chick embryos (obtained from 8-day-old infection free birds) were removed from the eggs and, after tissue disruption, were treated with 0.25% trypsin. The individual cells so produced were counted using the improved Neubauer haemocytometer. The cells were diluted with the growth medium (consisting of 0.5% lactalbumin hydrolysate and 5% cow serum in Hank's BSS) so as to contain approximately 4.8×10^4 cells/ml. 1 ml quantities of this cell suspension were then planted in screw-capped culture tubes and incubated. After 48 h, the growth medium was decanted and replaced with 1 ml of the growth medium containing 0.0 (control) to 0.10 ml of the root extract (1 mg of extractive/ml). The cultures were reincubated and examined every 24 h for cytopathogenicity

The degree of cytotoxicity was determined by microscopic examination of the cells and is expressed in form of a ratio of damaged to normal cells. The lowest level of the extract causing a 50% reaction was regarded as the toxic dose (TD 50)

Method 2. Doses of the extract (0.0–0.10 ml) were inoculated into the chorioallantoic sac of 15-day-old duck and chick embryos. The eggs were incubated at 37 °C using a Western Curfew Incubator under appropriate relative humidity setting (95%) and aeration. The eggs were candled daily to determine mortality rate. Viable eggs were allowed to hatch on the 21st day of incubation and the hatching eggs were examined for physical abnormalities

Counting of cells and estimation of growth. Following the method of Mirchamsy *et al* [11] for counting, an increase in the number of cells per culture within 24 or 48 h of incubation indicated growth of the culture

The amount of protein in the culture was estimated by the method of LOWRY *et al* [10] as modified by OYAMA and EAGLE [13] Doses of the root extract which reduced the total cell protein per culture by 50% were designated as the 50% inhibitory dose (ID 50) This was calculated as described by LITCHFIELD and WILCOXON [9]

Whole Animal Toxicology Studies

The mice used in these tests are Vom s strain (originally Wistar strain)

Acute toxicity Mice weighing 25 g were used for acute toxicity tests via three routes of administration (oral, intraperitoneal and intravenous) of the aqueous extract (1 25 g of extractive/ml) Oral administration was via a stomach tube (a No 2 serum tube without point or bevel attached to a polythene tubing) inserted carefully through the animal s throat when required Intraperitoneal injections were given with an 18$^{3}/_{4}$ inch needle passing through the abdomen while held taut and avoiding the liver, kidney, spleen or bladder Intravenous injections were given through a tail vein The vein was dilated by placing the tail in warm water (45 °C)

Chronic toxicity (a) Eight mice (average weight 15 g) were fed with 1 25 g/kg body weight of the extract daily for 7 days and set aside for observation (b) Mice weighing 15 g were used 15 male 15 female, 7 pregnant and 4 nursing mother mice were fed the extract at a dose of 22 mg/kg body weight twice daily for 6 weeks and 10 male, 10 female, 6 pregnant and 2 nursing mother mice had similar volume of distilled water daily, as control for the same period General observations were made of morbidity and mortality In addition lungs and livers of disected mice were examined blind microscopically at intervals and at the end of the feeding period

Preliminary Clinical Trial

A clinical trial similar to the one earlier reported for testosterone [7] was devised 50 young individuals (2–10 years of age) who had painful episodes of sickle cell disease at least twice a month and each with a pain score [7] of not less than 25 a month were used

5 ml of the root extract (1 mg of extractive/ml) was administered orally thrice daily for 2 months This followed a preliminary 2 month period spent in assessing a base line clinical picture for each patient The treatment period was followed by another 2 month period in which a similarly tasting aqueous extract of *Massularia acuminata* (Yoruba name Pako Ijebu another root used like *Fagara* for cleaning the teeth locally) was substituted for the *Fagara* extract

Results

Embryo Toxicology Studies

Table I shows the growth of primary cell cultures of chick and duck embryos in relation to dose level of the extract It shows that the extract has no significant effect ($P < 0.01$) on the cell cultures and the embryonated eggs of the duck and the chick The chicks and ducklings hatching from viable eggs on day 21 of incubation were normal birds exhibiting no

Table 1 The toxic (TD 50) inhibitory (ID 50) and lethal (LD 50) doses of *Fagara* root extract tested on chick and duck embryos

	0 0-0 10 ml of extract		
	TD50	ID50	LD50
Cell culture			
Primary duck (whole embryo)	0 05	2.3 2.6	–
Primary chick (whole embryo)	0 09	3 8-4 1	–
Embryonated eggs			
Duck	–	–	0 1-0 15
Chick	–	–	0 18 2.3

teratogenized features Toxicity, due to the aqueous extract of this *F zanthoxyloides* root, is virtually non-existent in cell cultures of whole embryos

Whole Animal Toxicology Studies

Acute Toxicity

Oral No deaths were recorded in 10 mice when fed the water-soluble extract, orally, at dose levels of up to 50 g kg

Intraperitoneal LD50 was obtained at 20 g/kg. At this dose level 60% of the mice had convulsion, only a third of these recovered from the convulsion the rest died The convulsions were of the clonic type and generally, the mice died of respiratory arrest Those that recovered were often depressed and hid away in a corner with a sagging head No ptosis was observed Mortality was inspected for 72 h

Intravenous LD50 was 8 g of extract kg body weight whilst all mice died at a dose of 14 g kg body weight Generally, there was increased respiratory movement with this LD100 dose initially, followed by a sedative or tranquillizing effect Reduced spontaneous activity and raised hind legs were observed before the animals died in a tonic convulsive state Urination was frequent

Chronic Toxicity

Eight mice, fed with 1 25 g of extract kg body weight daily for 7 days survived even after 60 days of observation Sets of mice fed 22 mg/kg body weight as well as those fed 1.25 g kg body weight for 7 days showed

Table II The effect of treatment with *Fagara* root extract on the pain score of sickle cell anaemia patients

Patient	Pain score while patient was treated with	
	control extract	*Fagara* extract
1	26	1
2	46	0
3	50	0
4	25	0

no pathological changes attributable to the test substance even after microscopical examination of livers and lungs

Preliminary Clinical Trial

This trial was plagued with a very high default rate Three types of data were obtained Firstly, 4 patients who had a pain score of 25–50 per month while on the control root (*Masularia acuminata* G Don) extract had a zero score per month on the *Fagara* extract (table II) Secondly, 4 patients who defaulted after being switched to the control extract from *Fagara* extract had a zero per month score whilst on *Fagara* Lastly, patients treated with *Fagara* extract who after a preliminary observation period refused to be put on a cross-over trial but who on their own took *Fagara* extract reported a complete and salutory change in well being The haematocrits of all the patients remained fairly constant or rose whilst they were on the *Fagara* extract

Discussion

The root of *F zanthoxyloides* is one of the agents used to clean teeth, in the Western State of Nigeria, as a preventive measure against dental caries It is used by chewing the sticks prepared as described earlier [3] followed by brushing of the teeth with the fibrous end thus produced In many of these local communities, the more elderly inhabitants cleaned their teeth with only this material usually with unavoidable or often deliberate swallowing of the juice produced from chewing the root Clearly, it would be expected that some documentation of side-effects would have

been made if any significant toxicity of the root extractive existed. In fact, it is still chewed today by many members of the present generation in this locality. However, since this local use has never been subjected to a scientifically organized toxicological testing, it was thought necessary to organize the toxicity tests here described. These have been reasonably comprehensive in the species studied including as they did studies in male, female, pregnant and embryonic forms as well as testing for both acute and chronic toxicity. The results confirm the local evidence, though circumstantial, of an absence of toxicity in the accepted usage of the root extract. Tests on primate species including subtle tests on functional capacity of treated animals will be carried out but this initial analysis of the toxicity status of the root extract is reassuring.

The clinical testing is in many ways preliminary. It was possible to take advantage of the HbSS individuals from poor, socio-economic environments who in the first 5 years get crises very frequently (e.g. one in a week or fortnight). Hence, any significant changes would be highlighted within a few months. Comprehensive clinical trials are being organized in a multicentric fashion and on a national scale spreading over the wet and dry seasons. This projected trial will give an authoritative appraisal. The limited *in vivo* examination here reported suggests, however, that there are grounds for cautious optimism that this root extract might secure a place in our armamentarium for managing sickle cell disease.

Acknowledgement. The authors wish to thank Mr. K. M. Jack and Mr. Dotti Akpewusi for technical assistance. We are grateful to the University of Ife for a research grant, to Tropical Products Institute, London, for an extra mural research grant, and to the Pharmaceutical Society of Nigeria – Sickle Cell Anaemia Research Fund, for financial support. This research is also financed by the OAU STRC.

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The *in vitro* Effect of Analgesics on the Electrophoretic Pattern of the Red Cell Membrane Proteins

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Abstract. The *in vitro* effect of acetylsalicylic acid, phenylbutazone and indomethacin on the electrophoretic pattern of the red cell membrane proteins is described by using the vertical urea starch gel electrophoresis. Normally 8 bands are visible. Acetylsalicylic acid and phenylbutazone bring about the disappearance of 5 bands and indomethacin that of 6 bands.

Key Words
Acetylsalicylic acid
Analgesics
Erythrocyte membrane proteins
Indomethacin
Phenylbutazone

The analgesics act on the red cells and on the hematopoietic tissue generally, causing a variety of abnormalities as aplastic, hemolytic or dyserythropoietic states [5, 8, 15, 16]. The present study describes observations on the effect of acetylsalicylic acid, phenylbutazone and indomethacin on the electrophoretic pattern of the red cell membrane proteins.

Materials and Methods

Red cells were collected from normal donors into ACD, washed 3 times in 0.9 percent NaCl and used on the day of collection.

For the *in vitro* action of the analgesics on the red cells, the method of Ma[illegible] and Smith [10] has been adopted. Cells were washed once with an isotonic (0.08 M, pH 7.4) phosphate buffer containing 6 mM glucose. The experimental system consisted of 5 vol of red cell suspension plus 2.5 vol of a solution of the drug. The PCV of the red cell suspension in the isotonic phosphate buffer was 25% and the PCV of the experimental system was 12.5%. Drugs were dissolved in phosphate buffer and the pH adjusted to 7.4 with either NaOH or HCl when necessary. The concentration of each drug in the solution is given in table I. The drug red cell

Table I Concentration, chemical characteristics and solvent of acetylsalicylic acid, phenylbutazone and indomethacin

Analgesic	Concentration mM	Formula	Molecular weight	Time of incubation h	Solvent
Acetylsalicylic acid	18	$C_9H_8O_4$	180 15	2 5	water
Phenylbutazone	2.4	$C_{19}H_{20}N_2O_2$	308 37	2 5	water
Indomethacin	9 5	$C_{19}H_{16}NO_4Cl$	357 78	2 5	water

mixtures were incubated at 37 °C under continuous agitation for 2.5 h in an Eberbach shaker

For the preparation of membrane proteins, the red cells were washed 3 times in isotonic phosphate buffer (310 mOsm, pH 7 4), 0 1 EDTA) All subsequent procedures were carried out at 4 °C. The cells were hemolyzed and washed in hypotonic phosphate buffer (20 mOsm pH 7 4, 0 1 EDTA) until the ghosts became white The membranes were sedimented after each wash by centrifugation at 30 000 *g* for 20 min The hemoglobin free ghosts were finally washed once with distilled water, resuspended in water and allowed to stand overnight [17] Solubilization of the protein was effected by the method of MADDY [9] An equal volume of *n* butanol (0 °C) was added to the membrane suspension, mixed well, allowed to stand for 30 min at 0 °C and then centrifuged at 30,000 *g* for 20 min The aqueous phase, containing the proteins, was removed with a cold syringe and dialyzed for 12 h against distilled water It was then lyophilized and stored under vacuum

Electrophoretic procedures 20 mg of the lyophilized protein powder were dissolved in 1 ml of a solution of 6 M urea, 3 percent acetic acid and 0 16 M 2 mercaptoethanol On standing overnight at 4 °C, a clear solution was obtained [1] The electrophoresis was carried out at an urea starch gel of pH 3 2 [14] The gel for this low-pH electrophoresis was prepared as follows 63 g of hydrolyzed starch was powdered together with 180 g of urea with pestle and mortar The gel buffer, consisting of 300 ml of sodium lactate buffer (pH 3 2, cation concentration 0 05 M) containing 2 4 ml 2 mercaptoethanol, was added to the powder and mixed before heating to 70 °C for 7 min in an Erlenmeyer flask After degasing, the gel was poured and allowed to set overnight The protein solution was subjected to electrophoresis for 6 h at a 80 mA constant current in a vertical system using the same sodium lactate buffer, pH 3 2, of the same cation concentration in the electrode chambers The gel was stained with amido black 10B and destained with a 3 percent acetic acid solution [13]

Results

The electrophoretic pattern of normal red cell membrane proteins consists of 8 bands [2] the slow-moving group of 3 bands (group c), a

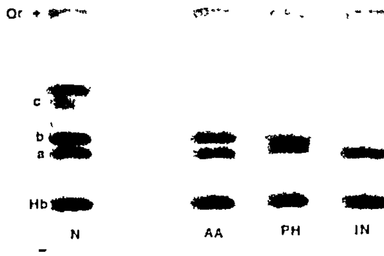


Fig. 1. Electrophoretic patterns of red cell membrane proteins treated with acetylsalicylic acid (AA), phenylbutazone (PH) and indomethacin (IN) and of normal (N). Or = origin. c = group of bands c. b = band b. a = band a. Hb = Hb band.

not always well defined band, 2 bands in the middle of the electrophoretic pattern (bands b and a) an other not always well defined band and finally, the fast moving band of hemoglobin (Hb) (fig. 1, N). The electrophoretic pattern of the red cell membrane proteins treated with acetylsalicylic acid consists only of bands b, a and the band of Hb (fig. 1 AA). After treatment with phenylbutazone it consists only of bands b and a which are very close one to an other, and of the Hb band (fig. 1, PH). After treatment with indomethacin it consists only of band a and of the band of Hb (fig. 1, IN).

Discussion

The electrophoretic study of the red cell membrane proteins [7, 11, 12] has been proved to be a useful method for the evaluation of alterations in various disease states [3, 4, 6]. The present experiments showed that acetylsalicylic acid, phenylbutazone and indomethacin, added *in vi-*

tro to erythrocyte suspensions, bring about abnormalities in the red cell membrane proteins, consisting in the disappearance of 5–6 bands out of 8 normally found The explanation and importance of these changes are not known Similar abnormal findings were observed on erythrocytes treated *in vitro* with oxidative agents and by substances producing PNH-like abnormality (unpublished observations)

Thus our findings seem to indicate that the above-mentioned analgesics bring about damage of the red cell membrane, apart from their known intracellular effect

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Phenylhydrazine (fig. 1). There is no proliferation of the marginal cells on the first 3 days. The marginal zone is 1–2 cells in thickness. There is no mitotic activity. On the 5th day, the thickness increases to a 4-cell layer. An occasional mitotic figure is seen. The proliferative activity is very short-lived, the thickness decreasing to a single layer on the 7th day.

Antiserum (fig. 1). The marginal cells do not show any proliferation on the first 2 days as with phenylhydrazine. On the 3rd and 5th day this layer varies from 2 to 4 layers in thickness. On the 7th day there is proliferation to 4–6 layers with occasional mitotic figures.

Discussion

From the present study it is evident that the RE cells of the marginal zone [3] around the lymphatic sheath proliferate in response to every type of hemolysis. After copper sulphate, there is a steady rise in the marginal zone thickness, reaching a peak on the 5th day. The responses with phenylhydrazine and antiserum are low. The hemolysis after copper sulphate is short-lived as compared with the other agents. Phenylhydrazine and antiserum produce a prolonged and sustained hemolysis [1]. Thus it is likely that hemolysis *per se* does not play an important role in marginal zone proliferation.

Copper sulphate produces a predominantly extravascular hemolysis, mainly due to erythrophagocytosis in the spleen [4]. Phenylhydrazine also produces a prominent splenic erythrophagocytosis [5]. As in this case, the erythrophagocytosis is sustained over a long period whereas with copper sulphate is short-lived, the marginal zone proliferation cannot be due to erythrophagocytosis as such. Moreover, the erythrophagocytosis with antiserum is much lower than with phenylhydrazine and yet both have a similar curve of marginal zone proliferation.

Studies on iron deposition in the splenic RE cells, show that 3 zones take part in the phagocytosis and breakdown of damaged red cells [6]. These are the red pulp, the marginal zone cells and the cells around the germinal centre. After phenylhydrazine the activity is mainly in the red pulp, reaching a peak on the 5th day. The marginal RE cells do not show any erythrophagocytosis. With antiserum activity is seen in all 3 zones. The activity is around the germinal centres with copper sulphate, slower activity is seen within the red pulp and the lowest in the marginal zone, the activity being mostly on the first 2 days.

As the erythrophagocytosis by the marginal cells is highest after antiserum whereas the proliferation is most marked after copper sulphate, it can be said that the proliferation is not connected with this activity alone. Moreover, no marginal zone activity is seen after phenylhydrazine but the proliferation is similar to that after antiserum. Copper is known to cause a marked increase in the metabolism of cells [7]. It seems likely that this action produces in the RE cells an increase in mitotic activity with proliferation and blast transformation, in contrast to cell death in the stable cells.

Studies on copper deposition [8] show that copper is at first released into the splenic RE cells and thence transported to the liver via the splenic and portal veins. From the 2nd day onwards there is a gradual increase of copper in the liver, indicating that the RE cells of the spleen release copper from this time onwards. The peak activity is on the 5th day [8]. Thus it can be said that the proliferation of the RE cells follows the release of copper from the red cell agglutinates and metalloprotein complexes [9].

The proliferation of the RE cells becomes prominent on the 3rd day, reaching a peak activity on the 5th day with prominent mitoses. By the 7th day there is a fall in mitotic activity. At this stage, the perivascular lymphatic sheath is almost completely replaced by the RE cells of the marginal zone. It is seen that abundant plasmacytoid cells are formed with the RE cell proliferation. This reaction is seen both in immune response [2] and with red cell sequestration [3]. In the present study, it is evident that although sequestration causes a proliferation of the marginal zone cells, it is much more prominent with red cells damaged by copper.

As copper is non antigenic and is a normal constituent of all body cells, the hemolysis produced by it being a short lived extravascular type, proliferation seems to be due to a direct action of copper on the cell metabolism [7].

Acknowledgement: I am very grateful to the Director Principal of the Maulana Azad Medical College, Dr. S. Padmavati, for giving me permission to publish this work.

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Acta haemat. 53: 175–182 (1975)

Coexistence of β-Thalassaemia Trait and Gilbert's Syndrome in Three Families

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Abstract. Three young unrelated propositi had β thalassaemia trait (β th t) and chronic unconjugated hyperbilirubinaemia (UH). Red blood cell ^{51}Cr t/2 was slightly reduced (from 19.0 to 22.8 days), liver function and histology, and iron metabolism included ferrokinetics and iron-chelation with Desferrioxamine B were normal. Bone marrow smears did not show features of dyserythropoiesis. Out of 20 relatives with normal liver function, β th-t was present in 7, UH in 7, and β th t and UH in 3, while the remaining 3 subjects were normal. UH was chronic, benign, familial, without overt signs of haemolysis and with normal liver function, (and histology in 3 propositi) it was therefore of Gilbert's type. Bromsulphophthalein kinetics and the reduced caloric intake test confirmed the diagnosis.

Key Words
BSP kinetics
^{51}Cr RBC survival
Ferrokinetics
Gilbert's syndrome
Hyperbilirubinaemia
Thalassaemia

Heterozygous β thalassaemia is frequent in Italy, particularly the trait (β th-t). This is characterized by ... [illegible] ... 1.0 and 3.7 mg/100 ml has been reported in 7 out of 116 cases [4]. Haemolysis seems to be unimportant since RBC ^{51}Cr t/2 is normal or only slightly reduced [4, 7, 12]. A bone marrow source of hyperbilirubinaemia might be present, but normal morphology of red cell precursors, absent or very mild anaemia and almost normal ferrokinetics are found in most cases [4, 6, 7, 12]. Liver disease may obviously be present and cause hyperbilirubinaemia. However, in young subjects with β-th-t without anaemia and liver disease, the unconjugated hyperbilirubinaemia should be not automatically ascribed to the β-th t itself. In our region the Gilbert's syndrome, i.e. familial chronic benign unconjugated hyperbilirubinaemia without overt signs of haemolysis is not rare [3, 4]. The existence of β th t and Gilbert's syndrome may be hypothetically expected.

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Heterozygous β-thalassaemia is frequent in Italy, particularly the trait (β-th-t). This is characterized by microcytosis, decreased red cell osmotic fragility, elevated HbA$_2$ and absent or slight anaemia. Frank hyperbilirubinaemia is uncommon and elevation of unconjugated bilirubin between 1.0 and 3.7 mg/100 ml has been reported in 7 out of 116 cases [4]. Haemolysis seems to be unimportant since RBC ^{51}Cr t/2 is normal or only slightly reduced [4, 7, 12]. A bone marrow source of hyperbilirubinaemia might be present, but normal morphology of red cell precursors, absent or very mild anaemia and almost normal ferrokinetics are found in most cases [4, 6, 7, 12]. Liver disease may obviously be present and cause hyperbilirubinaemia. However, in young subjects with β-th-t without anaemia and liver disease, the unconjugated hyperbilirubinaemia should be not automatically ascribed to the β-th-t itself. In our region the Gilbert's syndrome, i.e. familial chronic benign unconjugated hyperbilirubinaemia without overt signs of haemolysis is not rare [3, 4]. The existence of β-th-t and Gilbert's syndrome may be hypothetically expected.

In this paper the previously unreported coexistence of β-th-t and Gilbert's syndrome documented with familial studies is described

Materials and Methods

Three unrelated propositi aged 17, 19 and 20 years come to our observation because of chronic scleral jaundice with otherwise normal clinical examination Family studies were made on 20 available relatives (fig 1, table I)

Haematological tests were done with current techniques unless specified and included Hb, PCV, MCH MCV, MCHC, RBC, WBC, reticulocytes and platelet counts, RBC osmotic fragility without and with 24 h incubation at 37 °C, ATP and glucose corrected autohaemolysis, ESR, peripheral and bone marrow smear examinations, Hb electrophoresis HbF [14] and HbA_2 [9] determination, pyruvic kinase and G 6-PD activity in RBC, iron and total iron binding capacity [2] bilirubin [8] (serum unconjugated bilirubin, SUB) and haptoglobin (commercially available immunoplates) in the serum

The following liver function tests were performed in all cases total and fractionated proteins, LDH α HBDH GO and GP transaminases pseudocholinesterasis, alkaline phosphatase and γGT in the serum oral cholecystogram Liver biopsy was done during laparoscopy in the propositi Prothrombin time, PTT and fibrinogen concentration were also available

In the 3 propositi RBC survival (^{51}Cr) was studied with technique C proposed by the International Committee for Standardization in Haematology [15] The bromsulphophthalein (BSP) clearance was obtained in 10 cases (including 3 propositi) after the i v injection of 5 mg/kg of dye (Merck) The true BSP retention (%) at 45 min was calculated taking as 100% the dye concentration at zero time Analysis of plasma dye disappearance curve by means of the technique of BARBER RILEY et al [1] gave transfer rate from plasma to liver (a), from liver to bile (h) and from liver back to plasma (b) Transfer rates are expressed in mg/mg/min Ferrokinetics [11] (2 cases) 24 h urinary iron output after the i m injection of 1 g Desferrioxamine B (Desferal Ciba) [5] bone marrow and liver tissue also stained with Perls' stain were available in propositi

In propositi and 5 of their relatives (3 of whom had raised SUB) the reduction of caloric intake to 400 a day was made for 24 h Serum bilirubin variations were recorded before and after the test according to OWENS and SHERLOCK [10] An increase of 100% or more in SUB suggests that hyperbilirubinaemia is due to Gilbert s syndrome

Results

In propositi both the features of β th t and unconjugated hyperbilirubinaemia were present (table I) β-th t and raised SUB were also found in some of the relatives (table I, fig 1)

Table 1 Haematological data and serum bilirubin in 23 subjects from 3 families

Subjects[1]	Age and Sex	Diagnosis[2]	Hb g%	RBC $\times 10^6/\mu l$	MCH pg	MCV μm^3	Ret %	RBC osmotic fragility[3]	HbA2 %	HbF %	Serum bilirubin mg% Total	Serum bilirubin mg% unconjugated
AI1	75 M	G	13.0	4.94	26	87	1.0	N	2.1	0.5	1.6	0.9
AII2	61 M	N	14.9	4.80	31	100	0.5	N	2.2	1.0	0.2	0.2
AII3	42 M	β	12.8	6.10	21	73	1.0	D	4.3	0.8	0.3	0.2
AII4	39 M	β	13.3	5.90	23	74	1.0	D	4.7	0.6	0.3	0.2
AII5	42 F	G	14.9	4.60	32	90	1.0	N	2.9	0.3	1.8	1.2
AII6	50 M	β+G	14.4	6.55	22	77	1.8	D	4.1	1.0	2.6	1.7
BI1	60 M	G	13.0	5.10	29	88	1.0	N	1.9	0.7	1.6	1.0
BI2	57 M	G	12.7	4.20	30	90	0.5	N	2.3	0.4	1.9	1.4
BI3	45 F	β	14.6	7.20	20	75	0.6	D	6.0	1.0	0.4	0.3
BII4	26 M	β+G	12.9	6.45	20	64	0.8	D	5.6	1.0	1.6	1.4
BII5	23 M	β+G	12.4	5.38	21	66	1.2	D	6.1	1.3	1.8	1.4
BII6	21 F	β	11.3	5.70	20	65	0.5	D	5.9	0.4	0.8	0.5
BII7	18 M	β+G	12.8	6.53	20	63	1.0	D	6.5	0.6	1.8	1.6
BII8	17 M	β+G	13.7	6.60	21	59	0.6	D	5.7	1.2	2.0	1.7
BII9	15 M	G	15.7	5.53	28	87	0.5	N	2.3	1.0	2.3	1.8
CI1	51 M	β	13.2	6.28	21	68	1.0	D	5.1	0.8	0.5	0.2
CI2	44 F	N	12.9	4.00	32	95	0.3	N	3.0	0.6	0.5	0.3
CI3	49 M	β	13.1	5.60	23	70	0.7	D	6.1	0.6	0.6	0.3
CI4	40 F	G	13.1	4.77	27	88	0.4	N	2.1	0.3	2.2	1.7
CI5	43 M	G	14.2	4.40	32	88	0.7	N	2.0	0.6	2.8	2.1
CII6	24 F	β	11.3	5.25	22	66	1.5	D	5.4	0.8	0.7	0.5
CII7	22 F	N	15.4	4.10	37	96	0.5	N	2.1	0.8	0.6	0.4
CII8	19 F	β+G	11.2	5.10	22	68	1.2	D	5.8	0.4	2.8	2.1

[1] See Figure 1. AII6, BII7 and CII8 are the propositi.
[2] N = Normal. G = Gilbert's syndrome. β = β-thalassaemia trait.
[3] N = Normal. D = decreased.

In this paper the previously unreported coexistence of β-th-t and Gilbert's syndrome documented with familial studies is described

Materials and Methods

Three unrelated propositi aged 17, 19 and 20 years come to our observation because of chronic scleral jaundice with otherwise normal clinical examination Family studies were made on 20 available relatives (fig 1, table I)

Haematological tests were done with current techniques, unless specified and included Hb, PCV, MCH, MCV, MCHC, RBC, WBC, reticulocytes and platelet counts, RBC osmotic fragility without and with 24 h incubation at 37 °C, ATP and glucose corrected autohaemolysis, ESR, peripheral and bone marrow smear examinations, Hb electrophoresis, HbF [14] and HbA_2 [9] determination, pyruvic kinase and G 6-PD activity in RBC, iron and total iron binding capacity [2] bilirubin [8] (serum unconjugated bilirubin, SUB) and haptoglobin (commercially available immunoplates) in the serum

The following liver function tests were performed in all cases total and fractionated proteins, LDH, α HBDH, GO and GP transaminases pseudocholinesterasis, alkaline phosphatase and γGT in the serum, oral cholecystogram Liver biopsy was done during laparoscopy in the propositi Prothrombin time PTT and fibrinogen concentration were also available

In the 3 propositi RBC survival (^{51}Cr) was studied with technique C proposed by the International Committee for Standardization in Haematology [15] The bromsulphophthalein (BSP) clearance was obtained in 10 cases (including 3 propositi) after the i v injection of 5 mg/kg of dye (Merck) The 'true' BSP retention (%) at 45 min was calculated taking as 100% the dye concentration at zero time Analysis of plasma dye disappearance curve by means of the technique of BARBER RILEY *et al* [1] gave transfer rate from plasma to liver ('a') from liver to bile (h) and from liver back to plasma (b) Transfer rates are expressed in mg/mg/min Ferrokinetics [11] (2 cases), 24 h urinary iron output after the i m injection of 1 g Desferrioxamine B (Desferal Ciba) [5] bone marrow and liver tissue also stained with Perls' stain were available in propositi

In propositi and 5 of their relatives (3 of whom had raised SUB) the reduction of caloric intake to 400 a day was made for 24 h Serum bilirubin variations were recorded before and after the test according to OWENS and SHERLOCK [10] An increase of 100% or more in SUB suggests that hyperbilirubinaemia is due to Gilbert's syndrome

Results

In propositi both the features of β-th-t and unconjugated hyperbilirubinaemia were present (table I) β-th-t and raised SUB were also found in some of the relatives (table I, fig 1)

Table II Iron metabolism and RBC ^{51}Cr t/2 in 3 cases with β-thalassaemia trait and Gilbert's syndrome

Tests	Normal values	Propositi's values		
		AIII6	BII7	CII8
Serum iron, μg/100 ml	100-150	125	129	119
TIBC, μg/100 ml	250-400	400	365	352
Perl's positive material				
Bone marrow		±	±	+
Liver tissue		±	±	+
Desferal test, μg Fe/24 h	1,000-1,500	1,590	1,210	1,320
^{59}Fe t/2, min	60-110	92	–	60
Utilization of ^{59}Fe for red cell production %	75-95	95	–	95
Haemoglobin synthesis g/day/l	1.3-2.5	2.9	–	1.4
^{51}Cr t/2, days	25-33	22.0	22.8	19.0

Morphology of bone marrow and ^{59}Fe and ^{51}Cr surface counting over sacrum, liver and spleen were normal

Table III BSP metabolism in 10 subjects from the 3 families, included propositi (AIII6, BII7, CII8)

Subjects	Diagnosis[1]	'True' retention at 45 min %	Transfer rates, mg/mg/min		
			a	h	b
AII4	β	1.76	0.1221	0.0578	0.0070
AII5	G	4.50	0.0901	0.0335	0.0051
AIII6	β+G	2.67	0.1056	0.0254	0.0090
BI2	G	3.71	0.0920	0.0407	0.0010
BI3	β	2.60	0.1420	0.0536	0.0078
BII4	β	1.87	0.1444	0.0570	0.0060
BII7	β+G	1.64	0.1027	0.0166	0.0027
CI3	β	2.70	0.1300	0.0530	0.0050
CI4	G	2.60	0.0950	0.0242	0.0116
CII8	β+G	0.58	0.1091	0.0348	0.0078
Normal values in 23 healthy subjects from		0.00	0.1196	0.0483	0.0002
to		2.57	0.2043	0.1552	0.0135

[1] β = β-Thalassaemia trait; G = Gilbert's syndrome

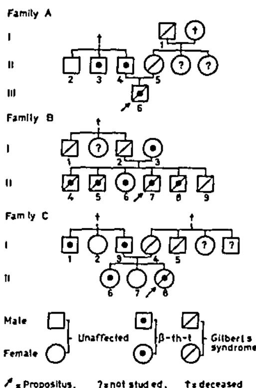


Fig 1 Family trees of the 3 propositi

In family A, raised SUB was present in maternal grand father (I1), mother (II5) and propositus (III6), β-th-t was found in father (II4), uncle (II3) and propositus

In family B, father (I2), uncle (I1) and brother (II9) had raised SUB, mother (I3) and sister (II6) had β-th-t The propositus (II7), and his brothers (II4, II5 and II8) had both raised SUB and β-th-t

In family C, uncle (I1), father (I3) and sister (II6) suffered from β-th-t, mother (I4) and maternal uncle (I5) had raised SUB The propositus showed both pathological conditions

Bone marrow examination did not reveal dyserythropoietic abnormalities, and Perls' stain was normal (table II) All other haematological tests, including serum haptoglobin, were normal Liver function and liver histology in propositi were normal

Iron metabolism and ^{51}Cr studies are shown in table II Serum iron, TIBC and percentage saturation of transferrin (30±6, mean ± 2 SD) were in the normal range Ferrokinetics, available in 2 cases, showed no

Table II Iron metabolism and RBC ^{51}Cr t/2 in 3 cases with β-thalassaemia trait and Gilbert's syndrome

Tests	Normal values	Propositi's values		
		AIII6	BII7	CII8
Serum iron, µg/100 ml	100–150	125	129	119
TIBC, µg/100 ml	250–400	400	368	352
Perl's positive material				
Bone marrow		±	±	+
Liver tissue		±	±	+
Desferal test, µg Fe/24 h	1,000–1,500	1,580	1,210	1,320
^{59}Fe t/2, min	60–110	92	–	60
Utilization of ^{59}Fe for red cell production, %	75–95	95	–	95
Haemoglobin synthesis, g/day 1	1.3–2.5	2.9	–	1.4
^{51}Cr t/2, days	25–33	22.0	22.8	19.0

Morphology of bone marrow and ^{59}Fe and ^{51}Cr surface counting over sacrum, liver and spleen were normal.

Table III BSP metabolism in 10 subjects from the 3 families included propositi (AIII6, BII7, CII8)

Subjects	Diagnosis[1]	'True' retention at 45 min, %	Transfer rates, mg/mg/min		
			a	b	b
AII4	β	1.76	0.1225	0.0578	0.0070
AII5	G	4.50	0.0801	0.0335	0.0031
AIII6	β+G	2.67	0.1056	0.0284	0.0050
BI2	G	3.73	0.0520	0.0407	0.0010
BI3	β	2.60	0.1420	0.0536	0.0075
BII6	β	1.87	0.1444	0.0570	0.0060
BII7	β+G	1.84	0.1027	0.0166	0.0027
CI3	β	2.70	0.1300	0.0530	0.0050
CI4	G	2.60	0.0950	0.0242	0.0116
CII8	β+G	0.58	0.1091	0.0345	0.0078
Normal values in 25 healthy subjects, from		0.00	0.1196	0.0493	0.0002
to		2.55	0.2045	0.1552	0.0135

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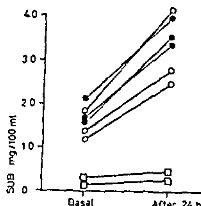


Fig 2 Effect of 24 h reduced caloric intake on SUB in 3 propositi with β th t and hyperbilirubinaemia (●) in 3 relatives with hyperbilirubinaemia (○) and in 2 relatives with normal bilirubinaemia (□)

abnormalities The RBC ^{51}Cr t/2 was only moderately reduced Surface counting (^{59}Fe, ^{51}Cr) was normal

BSP kinetics in 10 subjects (table III) showed that 'a' transfer from plasma to liver, 'h' transfer from liver to bile, and 'b' transfer from liver back to plasma were within normal range in subjects with β-th-t and normal SUB The transfers 'a' and 'h' were reduced below the upper limits of normal in subjects with raised SUB, regardless of the presence of β-th-t (AII5, AIII6, BI2, BII7, CI4 and CII8)

The reduced caloric intake tests (fig 2) indicated an increase between 90 and 133% in propositi, and 3 relatives with raised SUB only, and it gave normal results in 2 other relatives with normal SUB

Discussion

After having diagnosed β-th-t in the propositi, investigations on their unconjugated hyperbilirubinaemia were made Extensive studies on liver function and liver biopsy gave normal results POWELL *et al* [13] established that in subjects with normal liver function, only an RBC ^{51}Cr t/2 reduced below 15 days can cause hyperbilirubinaemia In our cases the ^{51}Cr t/2 was 22 0, 22 8 and 19 days, respectively Haemolysis as a possible cause of raised SUB was discarded Dyserythropoietic abnormalities were searched for Anaemia was absent, morphology of bone marrow was

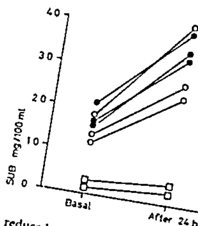


Fig 2 Effect of 24 h reduced caloric intake on SUB in 3 propositi with β-th t and hyperbilirubinaemia (●), in 3 relatives with hyperbilirubinaemia (○) and in 2 relatives with normal bilirubinaemia (□)

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normal and ferrokinetics showed no abnormalities Iron metabolism was also normal

As expected in each family β th t was present in one of the parents and also in some of the relatives However the parent without β th t and some other relatives had chronic unconjugated hyperbilirubinaemia In all relatives liver function was normal They had then a familial chronic benign unconjugated hyperbilirubinaemia without overt signs of haemolysis and with normal liver function The BSP clearance revealed some reductions in a (liver uptake) and h (excretion into bile) dye transfer rates like in Gilbert's syndrome [3] Moreover in the propositi and in 3 of their relatives with raised SUB the reduced caloric intake tests [10] indicated the presence of Gilbert's hyperbilirubinaemia In conclusion the hyperbilirubinaemia in the 3 families must be ascribed to Gilbert's syndrome Consequently the 3 young propositi suffering from β th t also had Gilbert's syndrome

We suggest that in β th t raised SUB must not automatically be considered as an obvious finding Only after having excluded liver disease haemolysis and dyserythropoiesis should Gilbert's syndrome be searched for

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Acta haemat. 53: 183–190 (1975)

Mikrokaryoblastenschub bei chronischer Myelose

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Abstract. After a 4 years' course a granulocytic leukemia terminated as a 'microkaryoblastic' leukemia. Morphology and cytochemistry of the microkaryoblasts are described.

Key Words
Granulocytic leukemia
Megakaryocytic leukemia
Microkaryoblast
Myeloproliferative disorders

In der Literatur wurden bisher nur wenige Fälle primärer megakaryoblastärer Myelosen mitgeteilt [7, 8, 10]. Dennoch wird eine Mitbeteiligung der megakaryoblastären Zellreihe bei myeloproliferativen Erkrankungen häufig gesehen [6]. So finden sich bei der Mehrzahl der Patienten mit chronischer Myelose im Knochenmark kleinere Megakaryozytenformen, vereinzelt sogar «Mikrokaryozyten» [1, 9]. Es wurde diskutiert, dass es sich bei diesen atypischen Zellformen um einen «Rückgriff in der Phylogenese» handeln könne [18]. Die Diagnose derartiger «Mikrokaryoblasten» kann erhebliche Schwierigkeiten bereiten und nur mit Hilfe zytochemischer und elektronenmikroskopischer Methoden gelingen. Wir berichten deshalb über einen terminalen Mikrokaryoblastenschub bei chronischer Myelose.

Methodik

Leukozyten, Thrombozyten sowie granulozytäre und thrombozytäre Vorstufen wurden aus heparinisiertem Venenblut gewonnen und für die elektronenmikroskopische Untersuchung präpariert [11]. Enzymcytochemische Untersuchungen wurden an Ausstrichen von Blut und Knochenmark durchgeführt: saure und alkalische Phosphatase [2, 13], Peroxydase [1[illegible], Naphthol-ASD-Chloracetatesterase [14

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Naphthol AS-Azetatesterase mit Hemmung durch NaF [12 17] Perjodsaure-Schiff Reagens [3]

Kasuistik

Patient Erwin K geb 17 10 38 1968 Diagnose einer chronischen myeloischen Leukamie typisches Blutbild und Sternalpunktat niedriger Index der alkalischen Leukozytenphosphatase Philadelphia Chromosom (Dr U KRUG Institut für Hama tologie, GSF Munchen) Behandlung mit Myleran (mit kurzen Unterbrechungen) bis 1972 1972 starke Grossenzunahme der Milz, vergrosserte Lymphknoten Auftreten atypischer Megakaryozyten in Knochenmark und Blut (Tab I) Thrombozyten 100 000/mm³ Philadelphia Chromosom bei erneuter Untersuchung weiterhin nach zuweisen Die Erkrankung sprach auf Myleran nicht mehr an Tod durch Verbluten aus einem Duodenalgeschwür Bei der Autopsie wurden diffuse myeloische Infiltrate aus atypischen Vorstufen der thrombo- und granulozytaren Zellreihe im untersuch ten Knochenmark in Milz und Leber sowie in den vergrosserten Lymphknoten nachgewiesen

Morphologische Befunde

Zytologie (1 2 1972) Die atypischen Megakaryoblasten (Mikrokaryo-blasten) in Blut und Knochenmark lagen oft herdformig zusammen Sie waren nur wenig grosser als Lymphozyten Ihre Kerne waren meist nie

Tabelle I Kernhaltige Zellen (%) in Knochenmark und Blut 1968 1972

	1968		1970		1972	
	Knochen mark	Blut (Leuko *22 000/* mm³)	Knochen mark	Blut (Leuko *46 000/* mm³)	Knochen mark	Blut (Leuko *100 000/* mm³)
Erythroblasten	12,5	–	6 5	–	26	7 5
Myeloblasten	3	1	1	2	2	8 5
Promyelozyten	7	5	5	13	3	7 5
(Meta) Myelozyten	37	7	40	17	4	10
Stab- Segm	37	69	44	58	18	21
Monozyten	1	5	–	5		3
Lymphozyten Plasmazellen	2	13	2 5	5	9	0 5
Mikro-Megakaryoblasten	0 5	–	1		38	42

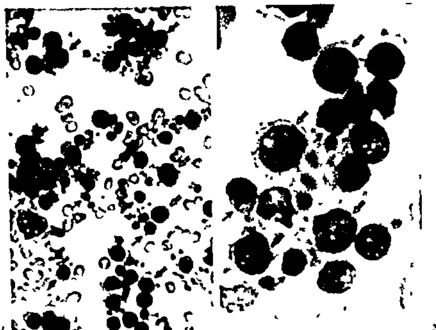


Abb. 1, 2. Sternalmarkausstrich. Mikrokaryoblasten mit rundlichen oder nierenförmigen, exzentrisch gelegenen Kernen und schmalem Zytoplasmasaum (Pfeile). Etwas grössere atypische Megakaryoblasten mit runden Kernen, lockerem Chromatin, eingefranstem Zytoplasmasaum (Doppelpfeile)

renförmig und exzentrisch gelegen, ihr Chromatin dicht; sie enthielten 0–1 Nukleolen. Das Zytoplasma erschien mittelblau und enthielt keine Granula. Die Zellgrenzen waren zum Teil unscharf. Gelegentlich waren die Zellen etwas grösser, ihr Zytoplasma heller (Abb. 1, 2).

Zytochemie (1.2.1972). Die Mikrokaryoblasten zeigten keine Aktivität von Peroxydase, von Naphthol-ASD-Chloracetatesterase oder von alkalischer Phosphatase. Sie wiesen eine starke Aktivität saurer Phosphatase auf, die besonders in der Kernbucht schollig-fleckig lokalisiert war. Naphthol-AS-Acetatesterase war deutlich positiv und durch NaF nur teilweise hemmbar. Besonders auffällig war eine deutliche Anfärbbarkeit mit der PAS-Reaktion: alle Zellen waren diffus angefärbt, einige zusätzlich ausgeprägt granulär.

Histologie (Prof. R. BURKHARDT, I. Med. Klinik, Universität Mün-

Naphthol AS Azetatesterase, mit Hemmung durch NaF [12, 17], Perjodsäure Schiff Reagens [3]

Kasuistik

Patient Erwin K, geb 17 10.38 1968 Diagnose einer chronischen myeloischen Leukämie typisches Blutbild und Sternalpunktat, niedriger Index der alkalischen Leukozytenphosphatase, Philadelphia Chromosom (Dr U KRUG, Institut fur Hämatologie, GSF München) Behandlung mit Myleran (mit kurzen Unterbrechungen) bis 1972 1972 starke Grossenzunahme der Milz, vergrosserte Lymphknoten, Auftreten atypischer Megakaryozyten in Knochenmark und Blut (Tab I), Thrombozyten 100 000/mm³, Philadelphia Chromosom bei erneuter Untersuchung weiterhin nachzuweisen Die Erkrankung sprach auf Myleran nicht mehr an Tod durch Verbluten aus einem Duodenalgeschwür Bei der Autopsie wurden diffuse myeloische Infiltrate aus atypischen Vorstufen der thrombo- und granulozytaren Zellreihe im untersuchten Knochenmark, in Milz und Leber sowie in den vergrösserten Lymphknoten nachgewiesen

Morphologische Befunde

Zytologie (1 2 1972) Die atypischen Megakaryoblasten (Mikrokaryoblasten) in Blut und Knochenmark lagen oft herdformig zusammen Sie waren nur wenig grosser als Lymphozyten Ihre Kerne waren meist nie-

Tabelle I Kernhaltige Zellen (%) in Knochenmark und Blut 1968–1972

	1968		1970		1972	
	Knochen mark	Blut (Leuko 22 000/ mm³)	Knochen mark	Blut (Leuko 46 000/ mm³)	Knochen mark	Blut (Leuko 100 000/ mm³)
Erythroblasten	12 5	–	6 5	–	26	7,5
Myeloblasten	3	1	1	2	2	8,5
Promyelozyten	7	5	5	13	3	7,5
(Meta) Myelozyten	37	7	40	17	4	10
Stab-, Segm	37	69	44	58	18	21
Monozyten	1	5	–	5	–	3
Lymphozyten, Plasmazellen	2	13	2 5	5	9	0,5
Mikro-, Megakaryoblasten	0 5	–	1	–	38	42

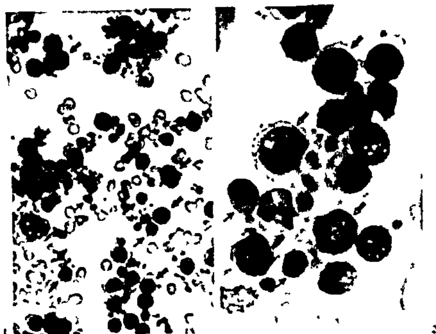


Abb. 1, 2 Sternalmarkausstrich. Mikrokaryoblasten mit rundlichen oder nierenförmigen, exzentrisch gelegenen Kernen und schmalem Zytoplasmasaum (Pfeile). Etwas grössere atypische Megakaryoblasten mit runden Kernen, lockerem Chromatin, sauggefranstem Zytoplasmasaum (Doppelpfeile)

renförmig und exzentrisch gelegen, ihr Chromatin dicht, sie enthielten 0–1 Nukleolen. Das Zytoplasma erschien mittelblau und enthielt keine Granula. Die Zellgrenzen waren zum Teil unscharf. Gelegentlich waren die Zellen etwas grösser, ihr Zytoplasma heller (Abb. 1, 2).

Zytochemie (1.2.1972). Die Mikrokaryoblasten zeigten keine Aktivität von Peroxydase, von Naphthol-ASD-Chloracetatesterase oder von alkalischer Phosphatase. Sie wiesen eine starke Aktivität saurer Phosphatase auf, die besonders in der Kernbucht schollig fleckig lokalisiert war. Naphthol-AS-Acetatesterase war deutlich positiv und durch NaF nur teilweise hemmbar. Besonders auffällig war eine deutliche Anfärbbarkeit mit der PAS-Reaktion: alle Zellen waren diffus angefärbt, einige zusätzlich ausgeprägt granulär.

Histologie (Prof. R. Burkhardt, I. Med. Klinik, Universität Mün

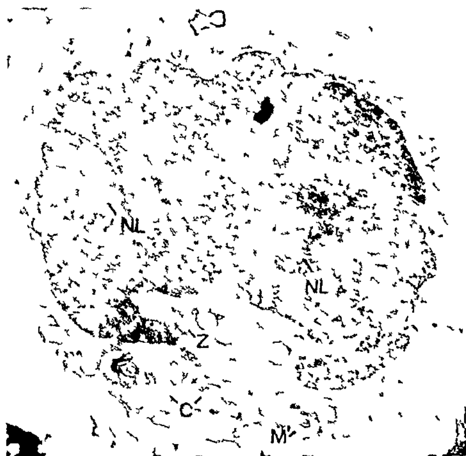


Abb 3 Mikrokaryoblast (entsprechend den mit Pfeilen gekennzeichneten Zellen der Abbildungen 1 und 2) Nierenförmiger Kern mit 2 Nukleolen (NL) Die Zellmembran bildet mikrovilliartige Ausstulpungen Im Zytoplasma zahlreiche Mitochondrien zum Teil mit Auftreibungen der Cristae oder Myelinfiguren (M') kleine Zytosomen (C) kurze Ergastoplasmaschlauche zahlreiche freie Ribosomen Zentrosom (Z) in der Zellperipherie Vakuolen × 11 700

Abb 4 Weiter differenzierter Megakaryoblast entsprechend den mit Doppelpfeilen markierten Zellen der Abbildungen 1 und 2 Im Zytoplasma zahlreiche kurze Ergastoplasmaschlauche und kleine Zytosomen diffus verteilte Glykogenpartikel links oben Bezirk mit kommunizierenden Vakuolen NL = Nukleole × 11 900

Abb 5 Zelle ähnlich Abbildung 4 Kommunizierendes Zisternensystem das «prospektiven Thrombozytenfeldern» gleicht N = Nukleus. × 23 000



5

chen, 3.2.1972). Die histologische Untersuchung eines durch Myelotomie gewonnenen Knochenzylinders zeigte eine dichte Infiltration des Markparenchyms mit atypischen myeloischen Zellen und hochgradig atypischen Megakaryoblasten, letztere zum Teil innerhalb der Sinuslumina. Vermehrung von Retikulinfasern.

Elektronenmikroskopie (1.2.1972). Die Mikrokaryoblasten sind rundlich, aber unregelmässig begrenzt (Abb. 3, 4). Die Zellmembran bildet zahlreiche mikrovilliartige Fortsätze. Die Kerne sind im Schnitt oval oder nierenförmig, sie enthalten 0–1–2 Nukleolen, das Chromatin ist gleichmässig verteilt ohne wesentliche Verdichtung entlang der Kernmembran, die Nukleolen zeigen zum Teil ausgeprägte Nukleolonemata, zum Teil sind sie von homogener Konsistenz. Das Zytosol ist dicht. Der Zelleib enthält sehr zahlreiche Mitochondrien, zum Teil mit Unregelmässigkeiten ihrer Cristae, zahlreiche kurze Ergastoplasmazisternen, freie Ribosomen und diffus (selten herdförmig) abgelagertes Glykogen. Selten sind kleine Granula. In der Zellperipherie finden sich häufig Vakuolen, die zu einem Zisternensystem konfluieren können, wie es sonst für die «prospektiven Trombozytenbezirke» reiferer Megakaryozyten typisch ist. Paranukleär waren bei einigen Zellen Bündel etwa 150 Å starker Fibrillen zu beobachten.

Diskussion

Im vorliegenden Fall handelt es sich um eine reifzellige Myelose, die nach 4jährigem Krankheitsverlauf das Bild einer megakaryoblastären Leukose mit Ausschwemmung kleiner, atypischer «Mikrokaryoblasten» ins periphere Blut bot. Die primäre Diagnose der reifzelligen Myelose ist gesichert durch typisches Blutbild, Knochenmarkbefund, Milztumor, niedrige alkalische Leukozytenphosphatase und Nachweis des Philadelphia-Chromosoms.

Die Diagnose eines «Mikrokaryoblastenschubs» einer chronischen Myelose ergab sich aus folgenden morphologischen Befunden: Elektronenmikroskopisch boten die Mikrokaryoblasten das Bild einer wenig differenzierten Zelle (Kernstruktur, weitgehendes Fehlen der für Megakaryozyten kennzeichnenden Zellorganellen). Sie zeigten fliessende Übergänge zu einer etwas grösseren Zelle, welche als Megakaryozytenvorstufe zu identifizieren war (einzelne kleine Granula, konfluierende Vakuolen, unregelmässige Zelloberfläche). Beide Zellarten waren kaum grösser als Lymphozyten (als welche sie auch zunächst im Blutausstrich fehlgedeu-

tet wurden). Die zytochemischen Befunde stützen diese Annahme. Die diffuse PAS-Positivität und die Naphthol AS-Azetatesterase-Aktivität grenzen die Zellen von Lymphozyten ab. Von Monozyten unterscheiden sie sich durch das vollständige Fehlen einer Peroxidaseaktivität (in der hier durchgeführten Methodik) und dadurch, dass die Naphthol AS-Azetatesterase nicht vollständig durch NaF zu hemmen ist. Gegen die Zugehörigkeit zur granulozytären Reihe sprechen das Fehlen von Peroxidase und Naphthol ASD-Chlorazetatesterase sowie die starke granuläre PAS-Positivität einiger Zellen. Lediglich bei verkürzter Glutaraldehydfixierung und längerer DAB-Inkubation lässt sich in Thrombozytenvorstufen Peroxidaseaktivität nachweisen [4, 5].

Die von uns beobachteten Mikrokaryoblasten zeigten Merkmale leukämischer Zellen: hohe Kern-Zytoplasma-Relation und eine Diskrepanz im Differenzierungsgrad von Kern und Zytoplasma, grosse Nukleolen, Vermehrung und strukturelle Besonderheiten der Mitochondrien, fibrilläre Zytoplasmastrukturen [11]. Eine Beteiligung der megakaryozytären Zellreihe am leukämischen Prozess ist daher auch nach diesen Befunden wahrscheinlich. Die Berechtigung des von DAMESHEK geprägten Begriffs der «myeloproliferativen Störungen» wird an diesem Beispiel demonstriert.

Bei niederen Wirbeltieren sind die gerinnungsfördernden geformten Elemente des Blutes kernhaltige diploide Zellen. Erst bei den Säugern kommt es zur polyploiden Entwicklung der Gerinnungszellen mit anschliessendem Zerfall des Zytoplasmas zu kernlosen «Thrombozyten» [15]. Es wird daher diskutiert, dass es sich bei den Mikrokaryozyten, welche gelegentlich bei hämatologischen Erkrankungen des Menschen zu beobachten sind, um einen «Rückgriff in der Phylogenese» handeln könne [18].

Zusammenfassung

Nach 4jährigem Krankheitsverlauf endete eine chronische Myelose mit einem «Mikrokaryoblastenschub». Morphologie und Zytochemie der Mikrokaryoblasten werden beschrieben.

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M. Bessis, R. I. Weed and P. F. LaCelle (eds.): Red Cell Shape. Physiology, Pathology, Ultrastructure. Springer, Berlin 1973. VIII + 180 pp., 147 fig., DM 32.40, US$ 12.00. ISBN 3-540-06257-2.

Es handelt sich um einen ausführlichen Bericht von einem 1972 abgehaltenen Symposium über Erythrozyten mit dem Schwergewicht auf ihrer Struktur und Form unter normalen und pathologischen Umständen. Die ausführlichsten Beiträge mit ausgezeichneten Abbildungen findet man im Sektor der Scanning-Elektronmikroskopie. Mit dieser Technik gelingt es dreidimensional strukturelle Veränderungen der Erythrozyten zu erkennen. Aufgrund dieser neuen morphologischen Erkenntnisse wird eine Reklassifizierung der pathologischen Erythrozytenformen vorgeschlagen.

Im zweiten Teil wird auf biochemische Faktoren eingegangen, die eine Veränderung der Erythrozytenform bewirken können. Plasmafaktoren und zelluläre Faktoren (unter anderen Alterung) werden ausführlich diskutiert.

Der dritte Teil ist hauptsächlich biophysischen Messungen, der Deformierbarkeit verschiedener Zelltypen und der Zellmembran gewidmet. Der letzte Teil setzt sich vor allem mit der Ultrastruktur von Sichelzellen, Innenkörper enthaltender Zellen sowie von Zellen, die durch Antikörper und Komplement beschädigt sind, auseinander.

Das Buch gibt neue Einblicke in die Ultrastruktur, Physiologie und Pathologie der Erythrozyten und ist eine wertvolle Erweiterung der bisherigen Literatur in diesem Sektor.

B. Speck, *Basel*

uation of labelled cells in the blood has several advan[illegible] for eventually harmful cell separations prior to or [illegible] labelling procedure, (2) no use of an unphysiological [illegible] ble elution of label from the lymphocytes, and (4) eval[illegible] logical level On the other hand the method of autotr[illegible] dine labelled lymphocytes as proposed originally [[illegible]] problem of utilisation of primarily not RNA bound label [illegible] sion The control of this source of error is the subject of [illegible]

Patients and Methods

Tritiated cytidine labelling was performed in 11 patients with [illegible] (cases A L) 2 patients with solid tumours (cases M and N) [illegible] oderma (case S) Cases A and B were patients in stage IV B of [illegible] pretreated with chemo and radiotherapy The pertinent data of [illegible] are reported a second paper [13]

Cell labelling and autotransfusion About 500 ml venous b[illegible] a transfusion bag (Fenwal Transfer Pack TA-4 Travenol, [illegible] being prevented by 75 ml ACD, formula A or – in patient L [illegible] (preservative free heparin, Hoffmann La Roche Grenzach) [illegible] cytidine (specific activity 2 1–4 0 Ci/mM Radiochemical Centre, [illegible] in case L, 1 mCi in all other cases) the blood was incubated for [illegible] in case L most of the unbound radioactivity (about 85%) was [illegible] the cells In all cases (including case L) after incubation the sp[illegible] cytidine was diluted approximately 1 000 fold by addition of [illegible] After that the labelled blood was autotransfused intravenously [illegible] ing a pressure of 100 mg Hg

Timing of blood sampling During autotransfusion two samples [illegible] the bag in order to determine the primary labelling of the [illegible] Within the 1st h after the end of autotransfusion six blood sam[illegible] a contralateral brachial vein of the patient, up to the 24th h further [illegible] to the 5th day four samples daily and later 1–2 samples daily

Preparation of the samples and autoradiography From each [illegible] WBC count was determined normal blood smears and smears of [illegible] trates (after sedimentation of the red cells with EDTA gelatine [illegible] of the leukocyte rich plasma supernatant) were performed. The [illegible] were fixed for 30 min with three times changed 100% methanol [illegible] autoradiography using Kodak AR 10 stripping film Long exposure [illegible] (mean 240) days were necessary to reach sufficient high label [illegible] than 9 grains/cell in about 85% of the incubated small lymphocytes [illegible] ment the autoradiographs were stained with 2% Giemsa solution [illegible]

Evaluation. In the blood smears the lymphocyte proportion of [illegible] was determined. Using this value and the WBC count the lymph[illegible]

Acta haemat. 53: 193–205 (1975)

Autotransfusion of ^{3}H-Cytidine-Labelled Blood Lymphocytes in Patients with Hodgkin's Disease and Non-Hodgkin Patients

I. Limitations of the Method[1]

P. Schick, F. Trepel, E. Lehmann-Brockhaus and H. Nietmann

I. Medical Division, Municipal Hospital München-Schwabing, and Division of Clinical Physiology, University of Ulm, Ulm

Key Words. Autoradiography · Hodgkin's disease · Lymphocyte kinetics · RNA metabolism

Abstract. Autotransfusion of ^{3}H-cytidine labelled blood lymphocytes followed by autoradiographic evaluation was studied in 11 patients with Hodgkin's disease and 3 other patients. It seems to be an appropriate method to estimate migration kinetics and pool sizes of lymphocytes. However, it is complicated by two major pitfalls: (1) The free (extracellular) activity of ^{3}H-cytidine transfused along with the cell bound label is sufficient to cause DNA labelling of newly produced lymphocytes in the body. (2) The soluble intracellular pool of ^{3}H labelled compounds causes an increase of labelling intensity of lymphocyte RNA in the first hours after cell transfusion. Methods to correct for these sources of error are described.

There is no knowledge about intravascular behaviour of blood lymphocytes and the size of extravascular lymphocyte pools in Hodgkin's disease. The method of transfusion of ^{3}H-cytidine-labelled autologous blood lymphocytes seems to be particularly suitable to provide quantitative estimations of exchangeable pools of lymphocytes, as has been demonstrated in patients with chronic lymphocytic leukaemia [2, 3, 5, 7, 9, 15].

Compared with the frequently applied method of autotransfusion of ^{51}Cr labelled lymphocytes and subsequent measurements of blood radioactivity, the autotransfusion of lymphocytes, labelled *in vitro* with the nucleic acid precursor ^{3}H-cytidine and subsequent autoradiographic eval-

[1] Supported by the 'Deutsche Forschungsgemeinschaft', SFB 112.

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For further information, contact EDWARD G BASSETT, Ph D, Symposium Coordinator, Miles Laboratories, Inc, *Elkhart, IN 46514* (USA)

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uation of labelled cells in the blood has several advantages (1) no need for eventually harmful cell separations prior to or immediately after the labelling procedure, (2) no use of an unphysiological label, (3) controllable elution of label from the lymphocytes, and (4) evaluation on the cytological level On the other hand the method of autotransfusion of ^{3}H-cytidine labelled lymphocytes as proposed originally [5, 7] introduces the problem of utilisation of primarily not RNA-bound label after transfusion The control of this source of error is the subject of this study

Patients and Methods

Tritiated cytidine labelling was performed in 11 patients with Hodgkin s disease (cases A L) 2 patients with solid tumours (cases M and N) and 1 patient with scleroderma (case S) Cases A and B were patients in stage IV B of Hodgkin s disease pretreated with chemo and radiotherapy The pertinent data of the other patients are reported a second paper [13]

Cell labelling and autotransfusion About 500 ml venous blood were collected in a transfusion bag (Fenwal Transfer Pack TA-4 Travenol Munich) coagulation being prevented by 75 ml ACD formula A or – in patient L – by 2,500 IU heparin (preservative free heparin Hoffmann La Roche Grenzach) After addition of ^{3}H cytidine (specific activity 2 1–4 0 Ci/mM Radiochemical Centre Amersham) (2 mCi in case L, 1 mCi in all other cases) the blood was incubated for 1–3 h at 37 °C. Only in case L most of the unbound radioactivity (about 85%) was eliminated by washing the cells In all cases (including case L) after incubation the specific activity of 'hot' cytidine was diluted approximately 1 000-fold by addition of 100 mg 'cold' cytidine After that the labelled blood was autotransfused intravenously within 8–11 min using a pressure of 100 mg Hg

Timing of blood sampling During autotransfusion two samples were taken from the bag in order to determine the primary labelling of the infused lymphocytes Within the 1st h after the end of autotransfusion six blood samples were taken from a contralateral brachial vein of the patient up to the 24th h further ten samples up to the 5th day four samples daily and later 1–2 samples daily

Preparation of the samples and autoradiography From each blood sample the WBC count was determined, normal blood smears and smears of leukocyte concentrates (after sedimentation of the red cells with EDTA gelatine and centrifugation of the leukocyte rich plasma supernatant) were performed The leukocyte smears were fixed for 30 min with three times changed 100% methanol and processed for autoradiography using Kodak AR 10 stripping film Long exposure times of 81–349 (mean 240) days were necessary to reach sufficient high labelling intensities of more than 9 grains/cell in about 85% of the incubated small lymphocytes After development the autoradiographs were stained with 2% Giemsa solution at pH 5 7

Evaluation In the blood smears the lymphocyte proportion of the leukocytes was determined Using this value and the WBC count the lymphocyte number per

microlitre blood was calculated In the autoradiographs of the leukocyte concentrates 4 000 small lymphocytes (nuclear diameter below 8.5 μm) were evaluated recording the number of silver grains over nucleus and cytoplasm of each cell The autoradiographic background of ³H free smears was below 2 grains/cell in 90% of the lymphocytes and did not exceed 9 grains/cell in any case Thus, in the experimental preparations, lymphocytes with more than 9 grains/cell were considered to be labelled

Results and Discussion

First survey The behaviour of labelled lymphocytes in the blood after autotransfusion is shown in the Hodgkin patients and non-Hodgkin patients (fig 1) In each of these patients the labelling index (LI) decreases quickly within the 1st h, and then stabilizes transiently at a very low level. After 4 h it begins to increase, reaching a first maximum after about 24 h and, interrupted by new decreases, further maxima at about 48 and 72 h During this period the curves of labelling intensity of the lymphocytes are nearly the reverse showing maxima at times when the LI are minimal Case L, who received an autotransfusion of washed labelled cells, shows initially a similar, but from 4 h onward a completely different labelling pattern (fig 2) The LI of lymphocytes, after a short rise from the 4th to the 12th h, decreases by the 24th h and thereafter shows only minor oscillations The labelling intensity increases during the first 6 h and then declines slowly until it reaches again the initial value at day 5

Since the labelling curves after transfusion of unwashed cells were similar in both groups, Hodgkin and non Hodgkin patients, the wavelike pattern cannot be interpreted as a Hodgkin's disease specific phenomenon As in case L, after transfusion of washed cells, the 24-hour periodicity of the LI is lacking and the labelling intensity in the first days exceeds the starting value, it is probable that in the other cases the 'free' activity of ³H-cytidine infused along with the cell bound activity was sufficient to cause an autoradiographically detectable additional *in vivo* labelling of lymphocytes This possibility was checked in a further experiment.

In vivo *utilization of ³H-cytidine* After 1–3 h of incubation less than 5% of the activity is incorporated in cells [unpublished data, 15] so that in our experiments more than 0 95 mCi 'free' activity are infused into the patient. In order to test the effect of the 1,000-fold dilution of the specific activity of the free ³H-cytidine case A received two infusions of 1 mCi ³H-cytidine of 9 min duration with an interval of 4 days. The specific activity of the first infusion of ³H-cytidine was 2.5 Ci/mM and that of the

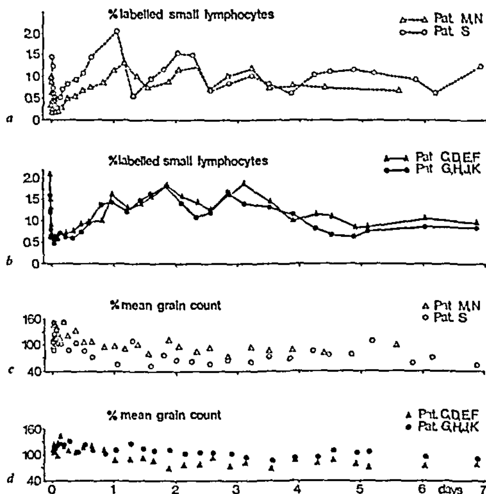


Fig 1 LI and labelling intensity of small lymphocytes after autotransfusion *A* LI in the non Hodgkin patients (values of cases M and N are pooled) *B* LI of the Hodgkin patients C–F (1 h incubation of lymphocytes with ^{3}H-cytidine) and G–K (3 h incubation) *C* Labelling intensity (all values expressed in percent of the mean labelling intensity at the time of autotransfusion) in the non Hodgkin patients *D* Labelling intensity in the Hodgkin patients

second infusion – by addition of 100 mg 'cold' cytidine – only 0 0025 Ci/mM Blood samples were taken from the patient at similar times as in the other cases after autotransfusions Part of the cell concentrate smears was extracted with 10% perchloric acid (PCA) for 36 h at 4 °C in order to remove macromolecular RNA from the cells [1] Extracted and non-extracted preparations were autoradiographed together and exposed for

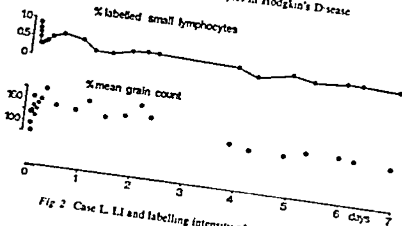


Fig 2 Case L. LI and labelling intensity of small lymphocytes.

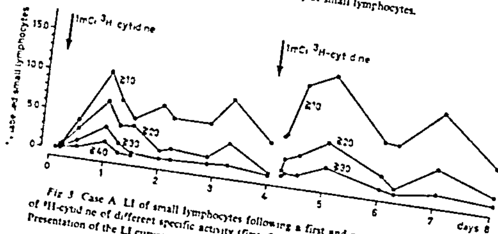


Fig 3 Case A. LI of small lymphocytes following a first and a second injection of ³H-cytidine of different specific activity (first 2.5 Ci mM second 0.0025 Ci mM) Presentation of the LI curves using different grain count thresholds.

300 days Figure 3 shows that small lymphocytes, labelled with more than 9 grains are found in the blood from the 4th h onward and increase to a maximum of 10.4% 24 h after the first injection of ³H-cytidine Some of these small lymphocytes were labelled with up to 55 grains After the second injection the LI increased again until the 24th h and reached, corrected for the rest labelling remaining from the first injection, 9.3%. The highest labelling intensity in some cells was 42 grains After RNA extraction similar labelling curves with only slightly lower values resulted maximal LI 8.9 or 8%, respectively, and maximal labelling intensity 40 or 30

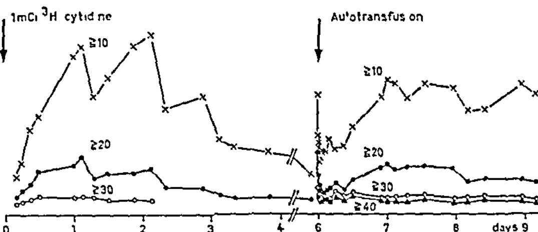


Fig 4 Case B LI of small lymphocytes following an injection of ^{3}H cytidine (left, specific activity, 0 0025 Ci/mM) and after autotransfusion of blood labelled with ^{3}H cytidine *in vitro* (right)

grains, respectively These findings show (1) that the i v injection of 1 mCi of ^{3}H-cytidine in man causes the labelling of a considerable proportion of small lymphocytes when long autoradiographic exposure times necessary for the autotransfusion studies are used, (2) that drastic dilution of the specific activity of ^{3}H-cytidine by the factor of 1,000 does not prevent such *in vivo* labelling of lymphocytes, and (3) that most small lymphocytes labelled *in vivo* by ^{3}H-cytidine are not labelled in their RNA (in contrast to *in vitro* labelling with ^{3}H-cytidine) but rather in other macromolecules, not extracted by PCA under these conditions, presumably in DNA This would mean that all labelled small lymphocytes in the PCA preparations are newly produced descendants from proliferating lymphoid cells which have actively incorporated ^{3}H-cytidine in their DNA The last interpretation is supported by observations in Hodgkin patients given a pulse injection of ^{3}H-thymidine [12] and showing a similar appearance and percentage of labelled small lymphocytes as after ^{3}H-cytidine injection described here

An additional experiment should test whether all 'free' activity in the preincubated autotransfused blood can be utilized *in vivo* like free ^{3}H-cytidine For this purpose case B received firstly an infusion of 1 mCi ^{3}H-cytidine with the low specific activity of 0 0025 Ci/mM and secondly, 6 days later, a usual autotransfusion of ^{3}H cytidine-labelled unwashed cells The maximal LI of small lymphocytes of 3 3% in the circulating blood

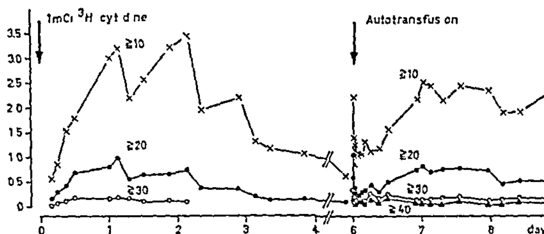
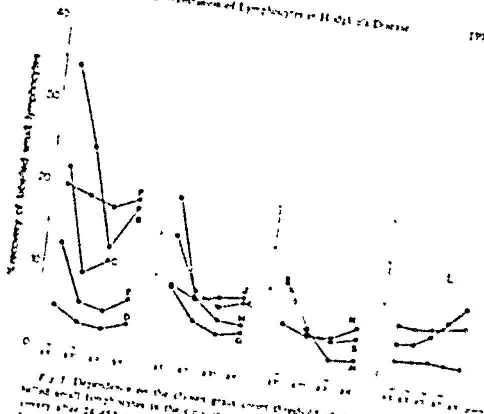


Fig 4 Case B LI of small lymphocytes following an injection of ^{3}H cytidine (left specific activity 0 0025 Ci/mM) and after autotransfusion of blood labelled with ^{3}H cyt d ne *in vitro* (right)

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Table I Patient B[1] Distribution of label during and after autotransfusion of ^{3}H-cytidine-labelled blood lymphocytes (ly)

Threshold of labelling intensity (grains)	LI of small ly in auto-transfused blood, %	Number of labelled small ly in autotransfused blood	LI in circulating blood, %[2]	Number of labelled small ly in circulating blood[2]	Recovery of labelled small ly in circulating blood %[2]
≥10	54 0	2 5 × 10^8	2 36	8 54 × 10^7	34 2
≥20	24 4	1 13 × 10^8	0 76	2 75 × 10^7	24 4
≥30	10 0	0 46 × 10^8	0 16	0 58 × 10^7	12 6
≥40	4 0	0 19 × 10^8	0 09	0 33 × 10^7	17 2

[1] Number of small lymphocytes/μl blood = 880, blood volume [estimated according to NADLER *et al*, 10] = 4 13 l, number of circulating small lymphocytes = 3 62 × 10^9, number of autotransfused small lymphocytes = 4 63 × 10^8

[2] Average of seven blood samples taken at different times between the 24th and 48th h after autotransfusion

Analytical procedure for separation of autotransfused lymphocytes from labelled newly produced lymphocytes In case B it is evident that a proportion of the autotransfused cells has a higher labelling intensity than the *in vivo* labelled newly produced small lymphocytes and that the limit is a grain count of about 30 (fig 4, table I) Since most newly produced lymphocytes are labelled with 10–20 grains, the true LI is most markedly falsified using a grain count threshold of 10 grains The error is less with a threshold of 20 grains and is practically excluded with a threshold of 30 grains

Under these conditions the 'recovery' (% recovered labelled cells after autotransfusion of labelled cells) in the circulating blood decreases step by step if the calculation of the recovery is based on increasing grain count thresholds (table I) The lowest recovery in patient B is to be expected at a labelling threshold of ≥ 30 grains As this is the case, the described procedure to look for the grain count threshold that reduces the recovery to the lowest value seems to be suitable to eliminate the admixture of labelled newly produced small lymphocytes in all other patients too (fig 5) The labelling curves, corrected on the basis of higher grain count thresholds are shown in figure 6 They now differ essentially from figure 1, but no longer from the labelling pattern in case L who had received washed cells (fig 2)

Table I Patient B[1] Distribution of label during and after autotransfusion of ³H-cytidine-labelled blood lymphocytes (ly)

Threshold of labelling intensity (grains)	LI of small ly in auto-transfused blood, %	Number of labelled small ly in autotrans-fused blood	LI in circulating blood, %[2]	Number of labelled small ly in circulating blood[2]	Recovery of labelled small ly in circulating blood %[2]
≥10	54 0	2.5 × 10⁸	2.36	8 54 × 10⁷	34 2
≥20	24 4	1 13 × 10⁸	0 76	2 75 × 10⁷	24 4
≥30	10 0	0 46 × 10⁸	0 16	0 58 × 10⁷	12.6
≥40	4 0	0 19 × 10⁸	0 09	0 33 × 10⁷	17 2

[1] Number of small lymphocytes/µl blood = 880, blood volume [estimated according to NADLER *et al*, 10] = 4 13 l, number of circulating small lymphocytes = 3 62 × 10⁹, number of autotransfused small lymphocytes = 4 63 × 10⁸

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Analytical procedure for separation of autotransfused lymphocytes from labelled newly produced lymphocytes In case B it is evident that a proportion of the autotransfused cells has a higher labelling intensity than the *in vivo* labelled newly produced small lymphocytes and that the limit is a grain count of about 30 (fig 4, table I) Since most newly produced lymphocytes are labelled with 10–20 grains, the true LI is most markedly falsified using a grain count threshold of 10 grains The error is less with a threshold of 20 grains and is practically excluded with a threshold of 30 grains

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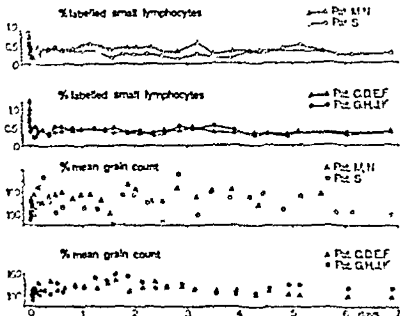


Fig. 6. LI and labelling intensity of heavily labelled small lymphocytes in Hodgkin and non Hodgkin patients (for details, see figure 1).

Changes of the labelling intensity of the in vivo labelled cells in vivo: optimal periods for calculation of the recovery. After 1 h incubation of 500 ml blood with 1 mCi ³H-cytidine the leukocytes contain only 1 or 5% of the activity [unpublished data, 15] and about 90% of this intracellular activity belongs to the soluble pool [4, 14]. Since the soluble pool is removed from the cells by methanol fixation [6], the autoradiographic labelling reflects the activity bound in macromolecules of the cell. The labelling intensity of newly synthesized RNA, however, depends on the concentration of ³H-cytidine in the soluble pool. This pool exchanges only slowly with the extracellular fluid [5, 11], so that 2 h after the end of ³H-cytidine exposure of the lymphocytes their soluble pool retains about [illegible] of its original maximal activity [14]. Therefore it can be expected that the lymphocytes labelled in vitro reach their maximal RNA labelling not during the 1 or 3 h of incubation but only some hours later, i.e. in our experiment some hours after autotransfusion. The increase of labelling intensity observed in most patients in the first hours after cell transfusion (fig. 1, 2,

Table II Patient L[1] Distribution of label *during* and after autotransfusion of ^3H-cytidine-labelled blood lymphocytes (*ly*)

Threshold of labelling intensity (grains)	LI of small ly in auto-transfused blood, %	Number of labelled small ly in autotransfused blood	LI in circulating blood, %[2]			Number of labelled small ly in circulating blood[2]			Recovery of labelled small ly in circulating blood, %[2]		
			5–30 min	20–48 h	5–7 days	5–30 min	20–48 h	5–7 days	5–30 min	20–48 h	5–7 days
≧ 10	95.2	9.19×10^7	0.54	0.38	0.23	6.91×10^6	4.86×10^6	2.94×10^6	7.52	5.30	3.20
≧ 30	80.1	7.73×10^7	0.44	0.33	0.19	5.63×10^6	4.22×10^6	2.43×10^6	7.29	5.46	3.15
≧ 50	55.5	5.36×10^7	0.31	0.28	0.14	3.97×10^6	3.58×10^6	1.80×10^6	7.40	6.69	3.35
≧ 70	35.6	3.44×10^7	0.21	0.23	0.09	2.69×10^6	2.94×10^6	1.10×10^6	7.81	8.56	3.20
≧ 90	22.2	2.14×10^7	0.13	0.17	0.05	1.66×10^6	2.18×10^6	0.67×10^6	7.90	10.20	3.13

[1] Number of small lymphocytes/μl blood = 270, blood volume [*estimated* according to NADLER *et al*, 10] = 4721, number of circulating small lymphocytes 1.28×10^9, number of autotransfused small lymphocytes = 9.65×10^7.

[2] Intervals after autotransfusion. The decreasing *concentration* of labelled lymphocytes in the blood with time after autotransfusion will be discussed later [3]

6) can be explained by this phenomenon. It is likely that the prolonged increase of labelling intensity causes a shift of *in vitro* labelled cells from lower into higher grain count classes, so that the distribution of the grain counts of the labelled cells *in vivo* differs from that evaluated in the autotransfused blood. Therefore, the recovery is overestimated, unless all infused small lymphocytes have been categorized as labelled. This overestimation should be low for instance in case L, where 95% of the transfused lymphocytes were labelled, if all labelled cells are considered. If, however, the labelling threshold is increased, the recovery values also increase as expected (table II). Also in the other patients, after elimination of lightly labelled cells representing mostly labelled, newly produced, not transfused cells, the recovery values increase with increasing grain count thresholds (fig. 5). About 5 days after autotransfusion the labelling intensity reaches again the starting values in the autotransfused blood and in the first samples in the circulating blood (fig. 5, 6). Now the recovery values are no longer dependent upon the labelling threshold applied. Hence it is assumed that the course of the labelling intensity curve indicates the optimal times for calculation of the true recovery from the LI of the more heavily labelled lymphocytes.

Conclusions

The LI of lymphocytes found in the circulating blood within the first 3 or 4 h after autotransfusion of ^{3}H-cytidine labelled blood can provide information on circulation kinetics of the cells if most autotransfused cells are labelled. The further course of the LI curve is falsified by the appearance in the circulating blood of lightly labelled newly formed lymphocytes, so that in this phase the behaviour of the labelled, originally transfused lymphocytes can only be observed selecting the more heavily labelled cells. Since, however, the labelling intensity of the *in vitro* labelled cells increases for some time following autotransfusion, an overestimation of the recovery of transfused cells can only be avoided if the calculations are based on the LI of heavily labelled cells after the labelling intensity has returned to the initial values.

Only taking into account these limitations, the method of analysing the recovery of autotransfused ^{3}H-cytidine labelled lymphocytes is suitable to estimate kinetic parameters of the traffic of lymphocytes in patients with Hodgkin's disease and other non leukaemic patients [13].

Acknowledgement We wish to thank Miss M MATZNER and Mr M EDER for diligent help in evaluation of the autoradiographs

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Autotransfusion of ^{3}H-Cytidine-Labelled Blood Lymphocytes in Patients with Hodgkin's Disease and Non-Hodgkin Patients

II Exchangeable Lymphocyte Pools[1]

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Key Words Autoradiography Hodgkin's disease Lymphocyte kinetics Recirculation of lymphocytes

Abstract The dilution in the circulating blood of lymphocytes labelled *in vitro* with ^{3}H-cytidine was examined after autotransfusion in 9 patients with Hodgkin's disease (HD) stage II A–IV B, 5 of whom were untreated, in 2 untreated patients with carcinoma, and in 1 treated patient with scleroderma. The blood transit time of exchangeable lymphocytes was 37±18 min in the patients with HD and 26±6 min in the other patients The proportion of exchangeable (recirculating) small blood lymphocytes was 39–84% in HD and 81–91% in the carcinoma patients. The relation between the size of the circulating pool of small blood lymphocytes and the total exchangeable (recirculating) lymphocyte pool was 1 20 to 1 30 in HD and 1·29 to 1 34 in the other patients The absolute size of the recirculating pool of lymphocytes was 46–90×10^9 cells in HD and 100–150×10^9 cells in the carcinoma patients

In Hodgkin's disease blood lymphocytopenia is characterized by a more or less pronounced decrease of the long-lived small lymphocyte population, whereas the short-lived lymphocytes are quantitatively normal or even increased [19, 21] Since long-lived blood lymphocytes are known to be part of the recirculating pool of lymphocytes [4, 5, 7, 8, 11, 17, 18], it could be supposed that this pool and other related exchangeable lymphocyte pools are diminished in correlation with the long-lived blood lymphocytes in Hodgkin's disease The present study attempts to

[1] Supported by the 'Deutsche Forschungsgemeinschaft', SFB 112

clarify this point using autotransfusions of blood lymphocytes labelled *in vitro* with ^3H-cytidine

Patients and Methods

In all, 9 patients with Hodgkin's disease (cases C-L), 2 patients with carcinoma (cases M and N) and 1 patient with scleroderma (case S) were examined. The pertinent clinical data are summarized in table I. The method of labelling and autotransfusion of lymphocytes and of the evaluation of radioautographic data is described in the preceding report [20].

Results

After incubation *in vitro* of 500 ml blood with 2 μCi/ml ^3H-cytidine for 1–3 h 64–95% of the lymphocytes were labelled with more than 9 grains (table II). The number of the autotransfused labelled (≥ 10 grains) small lymphocytes (N_0) varied from 0.92 to 7.37 × 10^8 (table III).

In all patients the concentration of labelled small lymphocytes in the circulating blood after autotransfusion decreases rapidly. The initial decline of the labelling index (LI) corresponds to a simple exponential function, characterized by a half time (fig. 1). The half times of disappearance of labelled lymphocytes were 13–45 min in the patients with Hodgkin's disease and 14–22 min in the other patients. By extrapolation of the slope of the disappearance curve towards the ordinate the LI for the time zero (corresponding to the end of autotransfusion) can be estimated to be 0.96–3.75% (table III). The LI of 0.87, 0.95 and 1.03% directly measured at time zero in cases L, M and N agree with the estimated values of 0.86, 0.95 and 1.17%, respectively. After the rapid decline in the first 30–45 min, the LI remained constant until the 3rd h. During this plateau phase the level of the LI in the single cases was rather different, varying from 0.19 to 1.0%.

Beyond the 3rd hour the LI increased [2?]. Beginning with that time the LI of the total labelled (≥ 10 grains) population, for methodological reasons, cannot be used for evaluation of lymphocyte traffic, because newly formed lightly labelled small lymphocytes arrive in the blood [20]. For quantitative considerations only the LI of more heavily labelled (≥ 30 grains) small lymphocytes are suitable [2?]. The LI of the more heavily labelled cells in the autotransfused blood are presented in table II; the

Acta haemat 53 206–218 (1975)

Autotransfusion of ^{3}H-Cytidine-Labelled Blood Lymphocytes in Patients with Hodgkin's Disease and Non-Hodgkin Patients

II Exchangeable Lymphocyte Pools[1]

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Key Words Autoradiography Hodgkin's disease Lymphocyte kinetics Recirculation of lymphocytes

Abstract The dilution in the circulating blood of lymphocytes labelled *in vitro* with ^{3}H-cytidine was examined after autotransfusion in 9 patients with Hodgkin's disease (HD) stage II A–IV B 5 of whom were untreated, in 2 untreated patients with carcinoma, and in 1 treated patient with scleroderma. The blood transit time of exchangeable lymphocytes was 37±18 min in the patients with HD and 26±6 min in the other patients The proportion of exchangeable (recirculating) small blood lymphocytes was 39–84% in HD and 81–91% in the carcinoma patients. The relation between the size of the circulating pool of small blood lymphocytes and the total exchangeable (recirculating) lymphocyte pool was 1 20 to 1 30 in HD and 1 29 to 1 34 in the other patients The absolute size of the recirculating pool of lymphocytes was 46–90×10⁹ cells in HD and 100–150×10⁹ cells in the carcinoma patients

In Hodgkin's disease blood lymphocytopenia is characterized by a more or less pronounced decrease of the long-lived small lymphocyte population, whereas the short-lived lymphocytes are quantitatively normal or even increased [19, 21] Since long-lived blood lymphocytes are known to be part of the recirculating pool of lymphocytes [4, 5, 7, 8, 11, 17, 18], it could be supposed that this pool and other related exchangeable lymphocyte pools are diminished in correlation with the long lived blood lymphocytes in Hodgkin's disease The present study attempts to

1 Supported by the Deutsche Forschungsgemeinschaft' SFB 112

clarify this point using autotransfusions of blood lymphocytes labelled *in vitro* with ^{3}H-cytidine

Patients and Methods

In all, 9 patients with Hodgkin's disease (cases C–L) 2 patients with carcinoma (cases M and N) and 1 patient with scleroderma (case S) were examined. The pertinent clinical data are summarized in table I The method of labelling and autotransfusion of lymphocytes and of the evaluation of radioautographic data is described in the preceding report [20]

Results

After incubation *in vitro* of 500 ml blood with 2 μCi/ml ^{3}H-cytidine for 1–3 h 64–95% of the lymphocytes were labelled with more than 9 grains (table II) The number of the autotransfused labelled ($\geq$ 10 grains) small lymphocytes (N_l) varied from 0.92 to 7.37×10^8 (table III)

In all patients the concentration of labelled small lymphocytes in the circulating blood after autotransfusion decreases rapidly The initial decline of the labelling index (LI) corresponds to a simple exponential function characterized by a half time (fig. 1) The half times of disappearance of labelled lymphocytes were 13–45 min in the patients with Hodgkin's disease and 14–22 min in the other patients By extrapolation of the slope of the disappearance curve towards the ordinate the LI for the time zero (corresponding to the end of autotransfusion) can be estimated to be 0.86–3.75% (table III) The LI of 0.87, 0.95 and 1.03%, directly measured at time zero in cases L, M and N, agree with the estimated values of 0.86, 0.95 and 1.17%, respectively After the rapid decline in the first 30–45 min the LI remained constant until the 3rd h During this plateau phase the level of the LI in the single cases was rather different, varying from 0.09 to 1.07%

Beyond the 4th hour the LI increased [20] Beginning with that time the LI of the total labelled ($\geq$ 10 grains) population, for methodological reasons, cannot be used for evaluation of lymphocyte traffic, because newly formed lightly labelled small lymphocytes arrive in the blood [20] For quantitative investigation only the LI of more heavily labelled ($\geq$ 30 grains) small lymphocytes are suitable [20] The LI of the more heavily labelled cells in the autotransfused blood are presented in table II, the

Table 1 Clinical data of patients

Patients	Age, years	Sex	Duration of symptoms	Clinical stage	Histologic classification[1]	Splenomegaly	Response to[2]			Lymphocyte count/μl	Pretreatment, free interval until this study
							PPD	DNCB	PHA		
Hodgkin's disease											
C	64	m	3 months	III A	MC	+	+	−	+	1 660	−
D	27	f	3 weeks	II A	MC	+	−	+ +	+ + +	1,070	−
E	26	m	3 months	II A	MC	−	−	n d	+	2 060	−
F	67	f	3 months	III B	MC	−	+ + +	−	−	1,240	−
G	76	f	4 years	III B	LD	+	−	−	−	1,200	chemotherapy, 2 months
H	33	f	7 years	IV B	LD	+	−	n d	−	930	chemotherapy, 2 days
J	70	m	3 years	III A	LP	−	+ +	+	+	2,200	local irrad, 2 years
K	20	m	1 month	III A	MC	−	−	−	+	1,460	−
L	49	m	8 months	IV B	MC	+ +	+ +	−	n d	670	local irrad, 6 months
Carcinoma											
M	66	m	6 months			+ +	−	n d	n d	700	−
N	65	m	1 year			+	+ + +	n d	n d	1,800	−
Scleroderma											
S	72	f	5 years			−	−	−	n d	1,390	chemotherapy, 1 year

[1] MC = Mixed cellularity, LD = lymphocyte depletion, LP = lymphocyte predominance

[2] PPD = Skin test with purified tuberculoprotein, DNCB = skin test with dinitrochlorobenzol, PHA = *in vitro* response of blood lymphocytes to phytohaemagglutinin (incorporation of ^3H thymidine) − = Negativ to + + + = normal range n d = Not done

Table II ^{3}H-Cytidine labelling of small lymphocytes (SL) *in vitro*

Patients	*In vitro* incubation period with ^{3}H-cytidine, h	LI of SL after incubation, %[1]	
		≳ 10 grains	≳ 30 grains
C	1	78.3	59.3
D	1	92.4	57.0
E	1	84.8	48.8
F	1	64.0	17.6
G	3	89.2	59.0
H	3	88.4	50.0
J	3	95.3	86.3
K	3	89.6	49.0
L	2	95.2	
M	3	94.0	80.1
N	3	94.4	80.2
S	1	88.1	46.5

[1] In cases C, J and N a grain count threshold of ≥ 20 was used, in case L no higher threshold was necessary [20].

corresponding absolute number of autotransfused labelled cells (N_{III}) and the LI in the circulating blood from the 5th to the 7th day after autotransfusion are given in table IV.

Discussion

If the circulating pool (CP) of small lymphocytes were a closed compartment it would be possible to calculate the LI expected in the circulating blood after autotransfusion of a definite number of labelled small lymphocytes (N_I) into a known pool of circulating lymphocytes (number of small lymphocytes/µl blood × blood volume in µl). The expected LI would vary from 7.19 to 14.42% (table III). Since, however, the initially observed exponential disappearance of labelled lymphocytes begins not only after but already during the autotransfusion, the expected LI derived from the above simple calculation must be overestimated. Assuming that all autotransfused labelled small lymphocytes are randomly eliminated from the circulation with a definite half time (fig. 1), the theoretically ex-

Table III Ratio of intravascular pool (IP) to circulating pool (CP) of small lymphocytes (SL)

Patients	Small lymphocytes/µl[1]	Blood volume, l	Circulating pool of SL (CP)	Labelled SL autotransfused (N_I)	LI excepted in CP (LI_I), [a]
C	1,190	5 45[2]	6 48 × 10⁹	4 89 × 10⁸	7 55
D	620	3 65[2]	2 26 × 10⁹	3 26 × 10⁸	14 42
E	1,320	4 92[3]	6 47 × 10⁹	5 13 × 10⁸	7 93
F	760	2 90[2]	2 20 × 10⁹	2 69 × 10⁸	12 23
G	640	3 16[3]	2 03 × 10⁹	2 84 × 10⁸	13 99
H	420	3 60[2]	1 51 × 10⁹	1 75 × 10⁸	11 59
J	1,590	4 25[2]	6 74 × 10⁹	7 33 × 10⁸	10 88
K	1,130	4 40[2]	4 97 × 10⁹	4 62 × 10⁸	9 30
L	270	4 72[3]	1 28 × 10⁹	0 92 × 10⁸	7 19
M	480	4 93[3]	2 35 × 10⁹	2 49 × 10⁸	10 60
N	1,520	3 60[3]	5 47 × 10⁹	7 37 × 10⁸	13 47
S	720	4 40[2]	3 18 × 10⁹	3 16 × 10⁸	9 94

[1] Note that small lymphocytes are only a fraction of all blood lymphocytes reported in table I

Table IV Estimation of the recirculating small lymphocyte (SL) pool (RP)

Patients	Heavily labelled SL autotransfused (N_{III})	LI expected in CP (LI_{III}), [a]	Heavily labelled long lived SL autotransfused (N_{IV})	LI expected in CP (LI_{IV}) [a]
C	3 71 × 10⁸	5 73	2 30 × 10⁸	3 55
D	2 01 × 10⁸	8 89	1 65 × 10⁸	7 30
E	2 95 × 10⁸	4 56	2 44 × 10⁸	3 77
F	0 74 × 10⁸	3 36	0 53 × 10⁸	2 41
G	1 88 × 10⁸	9 26	1 30 × 10⁸	6 40
H	0 99 × 10⁸	6 56	0 38 × 10⁸	2.52
J	6 64 × 10⁸	9 85	4 67 × 10⁸	6 93
K	2 53 × 10⁸	5 09	2 12 × 10⁸	4 27
L	0 92 × 10⁸ [c]	7 19	0 57 × 10⁸ [b]	4 45
M	2 12 × 10⁸	9 02	1 93 × 10⁸	8 21
N	6 26 × 10⁸	11 44	5 05 × 10⁸	9.23
S	1 67 × 10⁸	5 25	1 24 × 10⁸	3 90

[a] Mean of 3 5 values

[b] For calculation of maximal and minimal values, see text

Table III (continued)

Expected number of circulating autotransfused labelled SL (N_{III})[1]	LI expected in CP (LI_{III}), %	Calculated LI for zero time, %	Ratio of	
			LI_I to calculated LI	LI_{II} to calculated LI
4.54 × 10^6	7.01	1.72	4.39	4.07
2.52 × 10^6	11.15	1.84	7.84	6.06
4.26 × 10^6	6.58	2.36	3.36	2.79
2.19 × 10^6	9.95	3.75	3.26	2.65
2.55 × 10^6	12.56	1.14	12.27	11.02
1.12 × 10^6	10.73	1.52	7.63	7.06
6.64 × 10^6	9.83	1.80	6.04	5.47
3.72 × 10^6	7.48	3.00	3.10	2.49
0.80 × 10^6	6.25	0.86	8.36	7.27
2.03 × 10^6	8.64	0.95	11.16	9.09
6.32 × 10^6	11.55	1.17	11.51	9.87
2.75 × 10^6	8.65	1.70	5.85	5.09

[1] Measured by ¹³¹I albumin method.
[2] Estimated from body weight, height and sex [16].

Table IV (continued)

Observed LI in CP 5–7 days after autotransfusion, %	Ratio of		Size of RP[a]	
	LI_{III} to observed LI	LI_{IV} to observed LI	maximal values	minimal values
0.352	15.0	9.29	97.2 × 10^9	37.35 × 10^9
0.191	46.54	38.22	105.18 × 10^9	70.71 × 10^9
0.19?	23.27	19.23	150.56 × 10^9	102.85 × 10^9
0.300	11.2	8.03	24.64 × 10^9	12.63 × 10^9
0.150	51.44	35.56	104.42 × 10^9	49.75 × 10^9
0.116	56.55	21.72	85.39 × 10^9	11.08 × 10^9
0.532	18.52	13.03	124.82 × 10^9	61.76 × 10^9
0.3[illegible]	14.42	12.3	71.67 × 10^9	50.46 × 10^9
0.230	31.26	19.35	40.01 × 10^9	15.29 × 10^9
0.165	54.67	49.76	125.47 × 10^9	105.97 × 10^9
0.41?	27.57	22.24	150.81 × 10^9	95.09 × 10^9
0.25?	18.36	13.64	45.38 × 10^9	32.33 × 10^9

[a] Grain count threshold ≥ 10 grains (see table III).

pected number of labelled small lymphocytes in the CP (N_{II}) at the end of autotransfusion can be calculated as follows

$$N_{II} = \frac{N_I}{t \times \lambda} (1 - e^{-\lambda t})$$

where N_I = number of autotransfused labelled small lymphocytes, t = duration of autotransfusion, and $\lambda = \ln 2/T^1/_2$

According to this calculation 77 2–92 8% of the infused labelled cells should be in the CP at time zero (end of autotransfusion) That would correspond to LI (LI_{II} = N_{II} × 100/CP) of 6 25–12.56% (table III) As this calculation is based on the assumption that all infused labelled small lymphocytes are disappearing with the measured half times, the calculated values are minimum estimates of the expected LI This is about *the sixfold value of the LI (0 86–3 75%) observed in the circulating blood* at time zero (table III) Thus, most labelled infused lymphocytes leave the CP *during* autotransfusion in a time shorter than the half-times of disappearance observed *after* autotransfusion

One can argue that this is not a physiological phenomenon Killed lymphocytes are quickly eliminated from the circulation [6, 12] Since, however, after the trypan blue dye exclusion test more than 98% of the infused cells can be regarded as alive cell death is an unlikely explanation A rapid loss of labelling, that could simulate a rapid loss of labelled cells from the circulation, can also be excluded, for the labelling intensity of the lymphocytes does not decrease but rather increases after autotransfusion [21] We prefer two other explanations of the rapid disappearance of labelled cells during autotransfusion the intravascular trapping of labelled lymphocytes and/or the rapid mixture of the infused cells with an intravascular pool (IP) of lymphocytes which is larger than the CP Trapping of lymphocytes in non-lymphoid tissue after autotransfusion of ^{51}Cr-labelled cells in normal persons and in patients with chronic lymphocytic leukaemia does not seem to take place on a large scale [12, 15, 23] On the other hand, lymphocyte trapping in the patients under study cannot be excluded with certainty

On the premise that labelled small lymphocytes disperse homogeneously in the IP like granulocytes [1] the ratio of the expected LI (LI_I or LI_{II}, respectively) to the measured LI at time zero yields a maximum or minimum value respectively defining how much larger the IP should be than the CP (table III) In the Hodgkin patients the minimum values were 2 5–11 0, and the maximum values 3 1–12 3 Comparing with maximum

values of about 3 in haematologically normal person [2], the corresponding values in the four Hodgkin patients without splenomegaly (cases E, F, J, K) were similar (average 3.9 ± 1.4), in the other five Hodgkin patients with splenomegaly, however, considerably higher (average 8.1 ± 2.8).

While the rapid disappearance of labelled lymphocytes from the CP during autotransfusion could reflect the *dilution* in the larger IP, the random loss from the CP of labelled lymphocytes with half times of 15–45 min is interpreted to mean the efflux of small lymphocytes *from* the IP into an extravascular compartment. Similar half times of 15–30 min were observed after autotransfusion of normal blood lymphocytes labelled with ^{51}Cr [12] or normal thoracic duct lymphocytes labelled with ^{3}H-uridine [17]. Estimations of the blood transit time of lymphocytes ($T^{1}/_{2}$/ln 2) using another approach (exchange rate of radiologically damaged lymphocytes) amounted to maximally 60 min in man [6] and 20–40 min in calves [5]. The transit times calculated in our patients are on an average 37.5 ± 18 min in cases C–L and 26 ± 6 min in cases M, N and S. Since these values agree with the blood transit times mentioned above, it is concluded that the random loss of labelled small lymphocytes after autotransfusion reflects the emigration of recirculating cells from the intravascular pool into the extravascular part of the recirculating pool.

After the rapid exponential decrease in the 1st h after autotransfusion the LI becomes constant for at least 1 or 2 h (fig. 1). Since an influx of labelled small lymphocytes which could counterbalance a continued efflux is unlikely in this early phase it is concluded that the transient plateau of the LI is caused by the presence of labelled non recirculating small lymphocytes in the CP. Accordingly the maximal proportion of non-recirculating small lymphocytes (equal to the LI of the plateau phase divided by the LI of time zero) could be estimated to be 16–61.4% in the patients with Hodgkin's disease and 9.2–19.4% in the patients with carcinoma.

In rats, non recirculating lymphocytes are regarded as predominantly short lived [14, 25]. In uraemic patients the proportion of non recirculating lymphocytes of the thoracic duct lymph containing many short-lived cells was estimated to be about 17% [17]. On the basis of data from ^{3}H-thymidine labelling in vivo it was deduced that the proportion of short-lived small blood lymphocytes was about 10% in haematologically normal tumor patients (unpublished data) and 13.5–55% in patients with active Hodgkin's disease [22]. This is compatible with the hypothesis, that the non recirculating small lymphocytes in our patients are predominantly short lived. The mean life span of short lived small blood lymphocytes

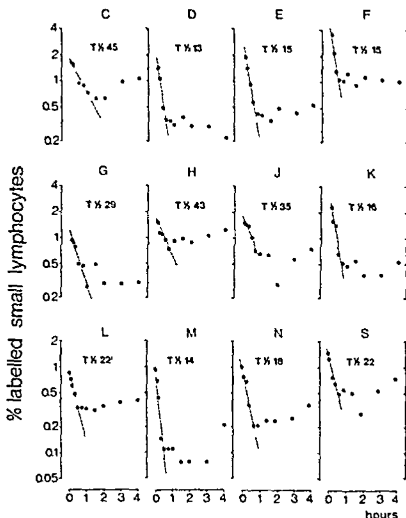


Fig 1 Semilogarithmic plot of the LI of small lymphocytes during the initial 4 h after autotransfusion C–L = Patients with Hodgkin's disease M N, S = non Hodgkin patients The half times of disappearance of labelled lymphocytes are indicated separately for each patient

was reported to be 4–7 days in rats and haematologically normal man [4, unpublished data] and 2 5–5 5 days in patients with Hodgkin's disease [21] Therefore it can be expected that most of the autotransfused short-lived lymphocytes are eliminated by the 5th day after autotransfusion For this reason and on methodological grounds [20] the LI of heavily labelled cells between days 5 and 7 are suitable for the estimation of the size of the recirculating pool of long-lived lymphocytes (RP), if the pro-

portion of long lived non recirculating lymphocytes is negligibly small. Taken this condition for granted, the ratio of the LI expected in a closed CP to the observed LI yields the factor by which the RP is larger than the CP. Maximum values of the expected LI (LI_{III}) are deduced from the total number of autotransfused heavily labelled small lymphocytes (N_{III}) in the following way: $LI_{III} = N_{III} \times 100$ CP. Minimum values (LI_{IV}) are expected if instead of N_{III} a number of autotransfused heavily labelled small lymphocytes reduced by the proportion of short lived lymphocytes[a] (N_{IV}) is used: $LI_{IV} = N_{IV} \times 100$ CP. The maximal factor by which RP is larger than CP is on an average 29.8 ± 17.5 in the patients with Hodgkin's disease and 33.5 ± 18.9 in the other patients, whereas the minimal values amount to 19.6 ± 10.9 and 28.6 ± 18.9 (table IV).

Similar values between 10 and 30 were estimated in patients without haematological disease and in normal animals [6, 13, 17, 18, 22]. Accordingly, the proportion of the RP represented by the CP with about 4% seems to be normal in Hodgkin's disease. The absolute size of the RP, however, is apparently reduced (table IV). The maximum values ($CP \times LI_{III}/LI_{observed}$) vary between 25 and 150×10^9 (average $89.3 + 39.5 \times 10^9$), the minimal values ($CP_{observed} \times LI_{IV}/LI_{observed}$) range from 11 to 103×10^9 (average $45.8 + 30.6 \times 10^9$). Compared to the size of the RP of the two carcinoma patients with $100–150 \times 10^9$ at the lower range of normal [24], the values indicate an average reduction of the RP to 35 and 64%, respectively, in the patients with Hodgkin's disease. In lymphopenic patients with chronic uraemia undergoing prolonged drainage of thoracic duct lymph the recirculating pool of small lymphocytes can be estimated to be $100–200 \times 10^9$ cells [17, 18], which would also correspond to the double value of that observed in patients with Hodgkin's disease.

If the IP is a homogeneous compartment, transit times of recirculating cells through CP and IP must be identical. Thus, the ratios of the minimal and maximal RP to the maximal and minimal IP multiplied by the transit time through the CP should yield minimum and maximum estimates of the mean transit times through the RP. These average recirculation times would be 2 and 3.4 h in the patients with Hodgkin's disease and 1.3 or 1.8 h in the other patients. These very short mean transit times, however

[a] Although the proportion of short lived cells was estimated on the basis of all labelled (≥ 10 grains) lymphocytes, it seems also to be valid for the heavily labelled cells, because the mean grain count did not change during the initial rapid decrease of LI after autotransfusion [illegible]

are compatible neither with the LI curves (fig 1) nor with data reported in the literature suggesting minimal transit times through the RP of 1–5 h [3, 7, 11, 17] and mean transit times of 6 or 27 h [7, 9]

Overestimation of the IP due to initial trapping of labelled lymphocytes must be considered as the most likely explanation of this discrepancy An initial trapping of transfused isogeneic thoracic duct lymphocytes lasting for less than 1 h has been described in rats, e g in the lung [10] The main consequence of a short term initial trapping of labelled auto transfused lymphocytes in the present study would be an overestimation of the IP The estimates of the blood transit time of exchangeable lymphocytes of the proportion of exchangeable and non exchangeable lymphocytes such as of the pool size of exchangeable lymphocytes (RP) should be correct The evaluation of these parameters depends on the fact that there is no permanent sequestration of damaged labelled cells This possibility is considered very unlikely, because autologous fresh anticoagulated whole blood without washing or centrifugation was transfused and because there was no difference of the recovery pattern of labelled lym phocytes after autotransfusion of blood cells incubated for 1 or 3 h

Acknowledgment We wish to thank Dr K BREMER (Department of Medicine University of Essen) who advised on the autotransfusion method Dr H CZEMPIEL (Nuclear Medical Division Mun cipal Hospital Schwabing) for measuring the blood volumes and Dr K H STEINBACH (Division of Clinical Physiology University of Ulm) The help in evaluation of the autoradiographs by Misses U DLASKA I DÖHR MANN E LEHMANN BROCKHAUS I OLSEN and K SCHMIDT RAUERT is gratefully appreciated

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Cellular and Subcellular Localization of Heme and Hemopexin in the Rabbit

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Key Words. Albumin · Autoradiography · Heme metabolism · Hemopexin · Liver ultrastructure

Abstract. The cellular distribution of intravenously administered ^{3}H heme, ^{125}I hemopexin and ^{131}I albumin was studied in rabbits. Radioautography of the tissue slides of several organs was examined by light and electron microscopy. All experiments were terminated 60 min after injection of the isotope labeled materials. ^{3}H heme and native monomeric ^{125}I hemopexin (heme saturated) were found to be associated with the endoplasmic reticulum and microbodies of the hepatocytes. Aggregated hemopexin was also taken up by macrophages of lung alveoli, splenic pulp, interstitial cells of the kidney and Kupffer cells. ^{131}I albumin (heme saturated) could not be located in liver, spleen, bone marrow, lung or kidney tissues.

Hemopexin, a serum β glycoprotein, serves as a carrier of heme in the blood [12]. It binds heme with a much greater avidity than albumin [16]. Heme is transported to the liver, where it is degraded mainly to bilirubin [14]. Previous work from our laboratory [8] has shown that the heme-hemopexin complex is removed exclusively by hepatocytes and not by the reticuloendothelial elements of the liver (Kupffer cells). In the present report, we have extended our investigations to the subcellular localization of ^{3}H heme and ^{125}I hemopexin. We have furthermore shown that aggregated hemopexin is taken up by the reticuloendothelial system throughout the body. In addition, we have shown that ^{131}I albumin is not removed by the hepatocytes during the process of plasma heme clearance.

Materials and Methods

Animals New Zealand white rabbits, 0.8–1.1 kg were purchased from Rancho de Conejo, Calif. Several days prior to the experiment, Lugol's solution was added to the drinking water.

^{3}H heme preparation Heme was labeled *in vitro* with ^{3}H aminolevulinic acid as previously described [11], the specific activity was 4×10^7 dpm/mg. Heme solutions were freshly prepared and 1 ml serum was admixed. Approximately 65 μg ^{3}H heme were injected.

Purification and isotope-labeling of proteins Rabbit hemopexin was purified from rabbits induced with lead acetate according to a previously described method [3]. Rabbit albumin was purified from plasma of healthy adult rabbits by preparative zone electrophoresis employing Pevikon as supportive medium [7]. Both proteins were tested for purity by immunoelectrophoresis and polyacrylamide gel electrophoresis [3].

Purified rabbit hemopexin and albumin were labeled with ^{125}I according to McConahey and Dixon [6]. The specific activity of the proteins was 7×10^8 dpm/mg protein. Rabbit serum, 0.2 ml, was added to each aliquot of isotope labeled protein and the mixture was centrifuged at 105,000 *g* for 2 h. This step was omitted when partly aggregated protein was used [9]. The amount of ^{125}I hemopexin injected was 3.8×10^8 dpm, that of albumin was 1.7×10^8 dpm. More than 97% of the isotope was protein bound when tested either by precipitation with 95% ethanol or with monospecific antibodies.

Experimental procedure Six rabbits were used. In two of them hemolysis was produced by administration of phenylhydrazine s.c. dissolved in phosphate buffered saline, pH 7.4 and administered (10 mg/kg body weight) every other day times five. Their hematocrit was 42 and 45% before treatment and 27 and 30% preceding the experiment, at which time intense reticulocytosis was present. Two animals were injected with either ^{125}I hemopexin or ^{3}H heme mixed with 1 ml serum. Two animals served as controls, i.e. they were not treated with phenylhydrazine, but were given either isotope labeled heme or heme-saturated hemopexin. Two additional animals, also untreated, received either ^{125}I methemalbumin or partly aggregated ^{125}I heme-hemopexin as previously described [9]. ^{125}I hemopexin was incubated with 0.4 mg hematin, an amount enough to saturate all circulating hemopexin, and ^{125}I albumin with 4 mg hematin for 30 min at room temperature before injection.

1 h after injection of ^{3}H heme or iodinated protein, the rabbits were sacrificed under anesthesia with sodium pentobarbital (Valley Veterinary Supply, North Hollywood, Calif.) 40 mg/kg body weight. Immediately following exsanguination through the abdominal aorta, liver, lung, kidney, spleen and bone marrow were removed. Small sections were taken for microscopic studies and autoradiography.

Microscopic and autoradiographic studies For light microscopy, the tissues were fixed in 10% phosphate buffered formaline and embedded in paraffin. Sections were obtained at 5 μm and processed for radioautography using NTB 2 (Kodak nuclear emulsion) [15]. The exposure time varied from 2 to 8 weeks. Sections were then lightly stained with hematoxylin and examined for localization of silver grains in the tissues. Several slides were studied for each exposure period.

For electron microscopy small pieces of tissue were fixed in 3% phosphate-buffered glutaraldehyde (pH 7.4) at 4 C. The tissue was then rinsed in buffer, post fixed in 1% cold OsO_4 similarly buffered After dehydration in graded alcohol it was embedded in epoxy resin Ultrathin sections in the range of silver were obtained with a Dupont diamond knife and placed on a Formvar-coated grid and processed for radioautography [15] using Ilford 3 emulsion (Essex, England) after an exposure time varying from 2 to 8 weeks. After each exposure period several grids were stained with uranyl acetate and lead citrate and studied in a Hitachi HU 11 A electron microscope for subcellular localization of radioactivity [15]

Results and Discussion

In a previous report, we had demonstrated that intravenously administered ^{3}H heme and ^{125}I hemopexin are removed by hepatocytes [8] The evidence was obtained by examining radioautographic tissue slides of several organs with the light microscope A tracer amount of ^{3}H heme was given as hematin, cyanmethemoglobin, methemalbumin or heme hemopexin Whatever pigment was injected, the heme was associated with liver parenchymal but not with Kupffer cells within 15–120 min of injection In the same study, tracer amounts of ^{125}I rabbit hemopexin were demonstrated to gain access to the hepatocyte The results of the present study confirm this conclusion

In contrast, ^{125}I albumin administered as methemalbumin, was not found in any organ studied This supports our recent suggestion that all hepatic ^{125}I albumin could be attributed to the plasma content of this organ [4] and is in agreement with results described by BISSELL *et al* [1] Even when a large quantity of hematin was given (12.5 mg/kg heme) the amount of ^{125}I albumin found in the liver did not increase [4]

By contrast in the experiment in which partly aggregated hemopexin was given silver grains were discernible both in hepatocytes and Kupffer cells (fig 1) Silver grains were also seen in the interstitial tissue of the lung and the kidney and in the cordal compartment of the splenic red pulp This distribution pattern suggests that the aggregated hemopexin was removed by the reticuloendothelial system. In this context, it is of interest to note that intravenously administered hemoglobin [13] and haptoglobin hemoglobin were found in cells of the reticuloendothelial system [17] Uptake of aggregated protein molecules may have been the cause of engulfment of these pigments BISSELL *et al* [1] located both entities only in hepatocytes

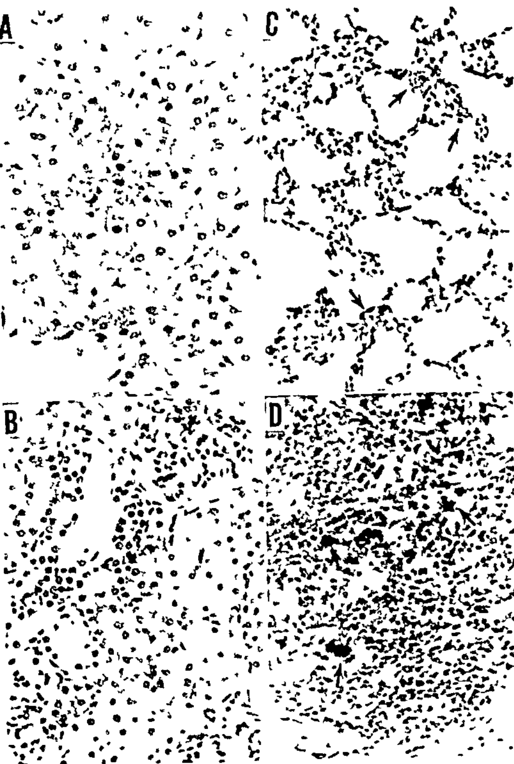

A
B
C
D



Fig. 2. Subcellular localization of ^{125}I hemopexin injected as heme hemopexin in a normal rabbit hepatocyte. The radioactivity is associated with rough and smooth endoplasmic reticulum and with microbodies (arrow). Similar distribution was found in phenylhydrazine treated animals. × 10,000

Fig. 3. Cellular distribution of aggregated ^{125}I hemopexin. Note that silver grains in the liver (A) are not confined only to hepatocytes but are also found in Kupffer cells. The radioactivity is seen in the interstitial tissues of lung (C, arrows) and kidney (B). In the spleen (D), the radioactivity is associated with the cordal compartment of the red pulp (arrows).

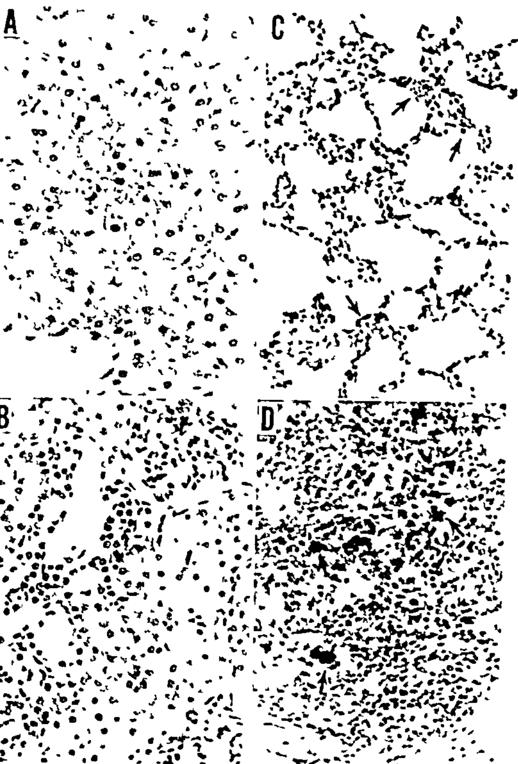
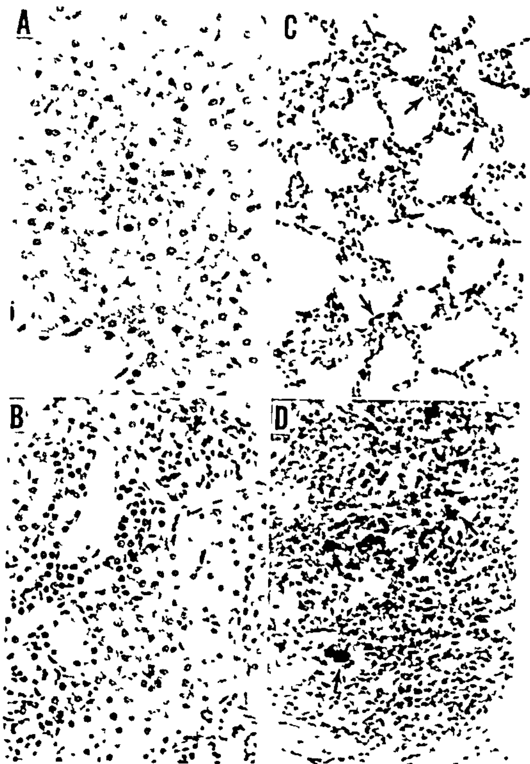

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Fig. 2. Subcellular localization of ^{125}I hemopexin injected as heme-hemopexin in a normal rabbit hepatocyte. The radioactivity is associated with rough and smooth endoplasmic reticulum and with microbodies (arrow). Similar distribution was found in phenylhydrazine treated animals. × 10 000.

Fig. 1. Cellular distribution of aggregated ^{125}I hemopexin. Note that silver grains in the liver (A) are not confined only to hepatocytes but are also found in Kupffer cells. The radioactivity is seen in the interstitial tissues of lung (C, arrows) and kidney (B). In the spleen (D) the radioactivity is associated with the cordal compartment of the red pulp (arrows).


A
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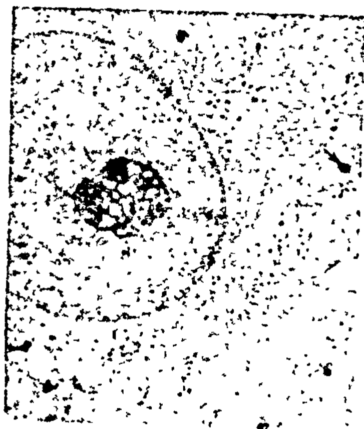


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[illegible] (arrows). Similar distribution was found
in phenylhydrazine treated animals. × 10,000

Fig. 1. Cellular distribution of aggregated [131I] hemopexin. Note that silver grains in the liver (A) are not confined only to hepatocytes but are also found in Kupffer cells. The radioactivity is seen in the interstitial tissues of lung (C, arrows) and kidney (B). In the spleen (D), the radioactivity is associated with the cordal compartment of the red pulp (arrows).

The finding that tracer doses of heme are taken up exclusively by liver parenchymal cells was confirmed in radioautographic studies by HERSHKO *et al* [2] who employed ^{59}Fe heme Their experiments also suggested heme to be entering the hepatocytes as the heme-hemopexin complex We have now verified these results by electron microscopy employing radioautography and ^{3}H heme as well as ^{125}I hemopexin We found the radioactivity after administration of both ^{3}H heme [10] or ^{125}I-hemopexin (fig 2) in the hepatocytes to be confined to the endoplasmic reticulum This was true both in normal and phenylhydrazine-treated animals A few silver grains were associated with microbodies No radioactivity was noted overlying plasma membrane, nucleus glycogen granules or mitochondria This subcellular distribution of heme hemopexin is in accordance with results obtained on the distribution of ^{3}H heme in microsomal, mitochondrial and cytosol fractions Within 45 min the major amount of ^{3}H was found in the microsomal fraction [4]

In view of the presented electron microscopic evidence and considering the much higer Kd of hemopexin than albumin for heme, we conclude that small amounts of heme are preferentially taken up by the hepatocyte as heme hemopexin Minute amounts of plasma heme have been considered to be naturally occurring repressors of microsomal cytochromes [5] This amount of heme is probably transported in the serum by hemopexin

Acknowledgements Supported by research grants HE 09660 from the National Heart Institute grant HD 04445 from the Institute of Child Health and Human Development and grant AM HL 16737 from the Institute of Arthritis and Metabolic Diseases and by Career Development Award AM 70551 to M TAVASSOLI from the National Institute of Arthritis and Metabolic Diseases and Career Development Award AM 16923 to U MULLER EBERHARD from the National Institute of Arthritis and Metabolic Diseases

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Acta haemat 53 226-229 (1975)

Erythrocytic Committed Hematopoietic Stem Cells in the Peripheral Blood of Mice

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Key Words CFU Erythrocytic commitment Hematopoietic stem cells

Abstract Hematopoietic stem cells (CFU) found in the peripheral blood of mice might be functionally, a heterogenous cellular population A comparison of our data with that of others leads to the conclusion that peripheral blood CFU belong to two or more marrow CFU subpopulations one of which consists of erythrocytic committed CFU

An approach to the problem of characterizing circulating hematopoietic stem cells (CFU) as being either multipotent [1] or unipotent [4, 5] is suggested by the recent finding that the hematopoietic organs of anemic Sl/Sl[1] mice are deficient in erythrocytic committed CFU [9] Apparently, the Sl/Sl[1] defect either disrupts the flow of multipotent CFU to the erythrocytic cell line or fails to support erythrocytic committed CFU proliferation It is believed that the granulocytic cell line continues to function normally in the presence of the defect [6, 9] and, although there is evidence that megakaryocyte maturation is altered [3] this apparently does not occur at the stem cell level [9] Therefore, a study of CFU migration in Sl/Sl[1] mice might afford one the opportunity of examining the contribution of a single committed stem cell compartment to the overall circulating CFU population In this communication data are presented characterizing erythrocytic committed CFU in the peripheral blood of mice and their mobilization into the circulation during periods of hematopoietic stress

Methods

Mutant Sl/Sl[1] mice and their normal congenic littermates (+/+) were obtained from the mating of WC/Re +/Sl females with C57BL/6J Sl[1]/+males at

the Jackson Laboratory, Bar Harbor, Me. Recipient B6WCF$_1$ (12–17 months old) used for the *in vivo* CFU assay were obtained by mating WC/Re males with C57BL/6J females at the AFRRI.

Blood was collected from the jugular vein of Sl/Sl^d and +/+ mice (10–18 weeks old) using 0.004 percent EDTA as an anticoagulant. Aliquots of pooled whole blood from two Sl/Sl^d or two +/+ mice were injected intravenously into each of 7–10 recipient mice which had previously received 950 rad of ^{60}Co whole body radiation. The mice were then housed 3 per cage and were allowed acidified water and food *ad libitum*. Nine days later, the mice were sacrificed, their spleens removed and fixed in Bouin's solution [8]. Gross nodule counts were made and, in some experiments, the spleens were sectioned (5 μm), HE stained, and the number of colonies determined microscopically.

Bodetella pertussis organisms in saline were purchased from Eli Lilly, Indianapolis, Ind.

Results and Discussion

As seen in table I, whole blood from +/+ mice injected into irradiated recipient mice generated twice as many macroscopic splenic colonies as an equivalent volume of blood taken from Sl/Sl^d donors. In an attempt to identify the missing circulating CFU in Sl/Sl^d mice, 5–6 spleens from the recipient mice were sectioned, stained and examined microscopically. It was found that 1 ml of +/+ blood generated approximately 98 microscopic colonies, 48 of which were erythrocytic, 20 granulocytic, 20 megakaryocytic and 10 mixed. Alternatively, blood from Sl/Sl^d donors generated only 78 microscopic colonies, 36 of which were erythrocytic, 24 granulocytic, 8 megakaryocytic and 10 mixed[1]. Thus, it would appear that the 'missing' peripheral blood CFU capable of generating macroscopic colonies are predominantly erythrocytic committed CFU, a finding which in some respects is consistent with the work of Bernstein [2] who reported that blood from Sl/Sl^d mice is only half as effective in curing the anemia of mutant W/W^v mice as is blood from +/+ mice.

It is noteworthy that peripheral blood CFU from both +/+ and Sl/Sl^d mice generated colonies whose hematopoietic type distribution was more characteristic of CFU from marrow than CFU of splenic origin [9]. Thus, the Sl/Sl^d defect does not appear to alter the previously

[1] On a per ml basis, marrow sections gave the following results: +/+ (Sl/Sl^d) control 45 (50) erythrocytic, 12 (12) granulocytic, 38 (6) mixed and 2 (2) megakaryocytic; 76 h, 100 (50) erythrocytic, 54 (44) granulocytic, 66 (5) mixed and 4 (16) megakaryocytic.

Table I Blood cellularity and CFU number of +/+ and Sl/Sl[d] mice following a single injection of *B pertussis* vaccine

Interval	Blood cellularity × 10⁶/ml	CFU/10⁶ cells	CFU ml of blood
+/+ mice			
Control	5 9 ± 1 9[1]	9 0 ± 1 2	50 ± 12
28 h	2.2 ± 0 6	12 8 ± 2 2	28 ± 4
76 h	10 4 ± 1 4	31.3 ± 4 4*	325 ± 51**
158 h	7 0[2]	18 2	131
Sl/Sl[d] mice			
Control	7 4 ± 1 8	3 6 ± 1 1	26 ± 10
28 h	–	–	–
76 h	6 2 ± 1 1	10 9 ± 1 9***	62 ± 5****
158 h	–	–	–

[1] Mean ± SEM of 2–4 determinations performed on 2 animals
[2] One determination on 2 animals
Significantly different from control at * $p<0.005$, ** $p<0.010$, *** $p<0.025$, **** $p<0.050$

suggested marrow origin of peripheral blood CFU [4], only the concentration of CFU committed to specific lines of hematopoietic cellular differentiation

CFU migration was further investigated by challenging both Sl/Sl[d] mice and their normal congenic littermates with a single injection (i p) of 20×10⁹ heat killed *B pertussis* organisms In normal mice, this treatment effects a rapid fall of marrow erythroid precursors and a sharp increase in the number of circulating CFU [7] In the present study, a 6 5-fold *increase in blood CFU concentration in +/+ mice* was observed, while in Sl/Sl[d] mice there was only a 2 4-fold increase (table I) The observed increase in +/+ mice was distributed among the various colony types This was not the case for Sl/Sl[d] mice Specifically, in the periphery of +/+ mice, CFU generating erythrocytic colonies increased 4 2-fold, granulocytic 2 5 fold, megakaryocytic 2 1-fold and mixed 3 5-fold The corresponding values for Sl/Sl[d] blood were as follows erythrocytic 0 34-fold, granulocytic 2 0 fold megakaryocytic 13 0-fold and mixed 4 0 fold Thus *B pertussis* vaccine treatment did not effect an increase in the number of circulating erythrocytic committed CFU in Sl/Sl[d] mice, only in +/+ mice

In summary, it appears that in Sl/Sl¹ mice, CFU capable of generating granulocytic and megakaryocytic colonies are released into the circulation independently of erythrocytic committed CFU, possible indicating that peripheral blood CFU are, functionally, a heterogeneous cellular population. Furthermore, in view of the work of GIDÁLI *et al.* [4] suggesting that peripheral blood CFU represents a subpopulation of the heterogeneous marrow CFU population, and the work of WOLF [9] demonstrating that Sl/Sl¹ marrow is deficient in erythrocytic committed CFU, it might also be concluded that peripheral blood CFU belong to two or more marrow CFU subpopulations, one of which consists of erythrocytic committed CFU.

Acknowledgements. We are grateful to Mr. GEORGE LEE and Mr. PETER KIRCHGESSNER for their technical assistance.

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Acta haemat 53 230–240 (1975)

Massive Splenomegaly, Pancytopenia and Haemolytic Anaemia in Sarcoidosis

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Key Words Haemolytic anaemia Hypersplenism Pancytopenia Sarcoidosis Spleen sarcoidosis

Abstract A 53-year-old dock labourer, presented with massive splenomegaly and subsequently developed pancytopenia Complete haematological remission was observed following splenectomy Histological examination of the liver and spleen revealed sarcoid granulomata A year after splenectomy, he died in an acute haemolytic crisis a very rare complication of sarcoidosis Evidence of generalized sarcoidosis was found at autopsy The literature on haematological complications in sarcoidosis is reviewed

Splenomegaly is a relatively uncommon feature of sarcoidosis In a series of 275 patients with sarcoidosis Scadding [35] recorded a palpable spleen in 31 instances In only 5 of these patients was the enlargement considerable and none had any symptoms attributable to this finding However, associated anaemia, leucopenia, thrombocytopenia, singly or in various combinations, have been described [4, 9, 10, 12, 14, 16, 18–21, 23, 24, 28, 31–34, 37, 43] Haemolytic anaemia is a very rare complication of sarcoidosis and to date we have been able to trace only 13 well-documented examples [2, 6–8, 13, 17, 22, 25, 26, 38, 41, 42 44]

We wish to report a patient with sarcoidosis who presented with massive splenomegaly and developed features of hypersplenism Following splenectomy he died as a result of an acute haemolytic crisis

Case Report

A 53 year old dock labourer was admitted to hospital on 28 7 1969 with a 3 year history of recurrent abdominal distension and recent weight loss 2 weeks

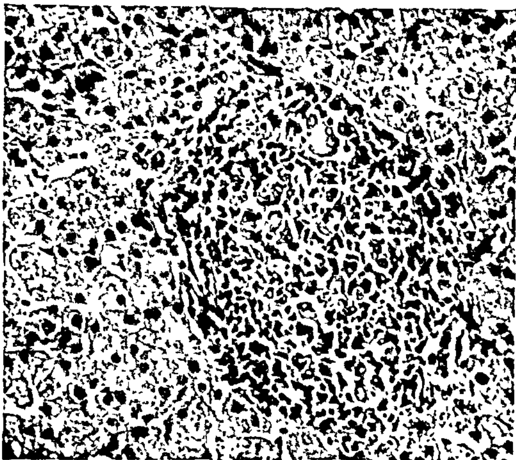


Fig 1. Photomicrograph of liver showing non-caseating epitheloid cell granuloma of sarcoidosis, containing multinucleated giant cells. HE × 360

bin, 7.6 g/100 ml, white cell count, 26,000 µl, and reticulocyte count 12%. The serum bilirubin was 5.2 mg/100 ml. The direct antiglobin test was strongly positive. The serum proteins and electrophoretic pattern were still within normal limits and fluorescent antibody tests for antinuclear factor were negative. The bone marrow examination revealed erythromyeloid hyperplasia. A diagnosis of acute haemolytic anaemia of warm antibody type was made, but before treatment could be started he died.

At autopsy (J. J. T.) the tissues were jaundiced. The liver now appeared macroscopically normal but histologically there were many lymphoid and reticulum cells in the portal tracts. The mediastinal and abdominal lymph nodes were visibly enlarged and histologically there was replacement of germinal follicles by lymphoid cells with some larger cells of reticulum cell type. The bone marrow, kidneys and liver contained multiple nodules which closely resembled the original liver biopsy in his

tological appearance. The lungs showed areas of organized and acute pneumonic changes. Guinea pig inoculation of the bone marrow taken at autopsy failed to show any abnormality.

Discussion

The diagnosis of sarcoidosis in this patient was made on the basis of typical histological findings, multiple organ involvement, and exclusion of other categories of granulomatous disease [36]. A negative Kveim test does not necessarily preclude this diagnosis [27, 40]. Massive splenomegaly as the presenting feature of sarcoidosis, although described previously [3, 5, 15, 18, 29, 45], is unusual and poses diagnostic problems. Enlargement of this organ, irrespective of the primary aetiology may be associated with the syndrome of hypersplenism [39] with the development of either a pancytopenia or anaemia, leucopenia and thrombocytopenia in various combinations. Great diagnostic problems were encountered in our patient who initially presented with massive splenomegaly and subsequently developed pancytopenia. Diagnosis of sarcoidosis was made only after histological examination of the liver and splenic tissue obtained at laparotomy. Hypersplenism in sarcoidosis leading to pan- or selective cytopenia is a rare but well-documented complication [4, 9, 10, 12, 14, 16, 18–21, 23, 24, 28, 31–34, 37, 43]. However, it is still not known why only a minority of patients with sarcoidosis who have enlarged spleens develop either a total or selective cytopenia. In the present case and in all the reported examples of cytopenic syndromes in sarcoidosis the spleen has been infiltrated with sarcoid granulomata.

It is well recognized that splenectomy can lead to amelioration of the haematological complications in sarcoidosis [1, 4, 5, 15, 21, 28, 30, 33, 37]. Our patient was unusual in that he remained clinically well for 1 year following splenectomy and then died in an acute haemolytic crisis. However, in 1956 LEBACQ *et al* [22] also reported a 23-year-old girl in whom splenectomy performed for the cytopenic syndrome affecting the white and red cell series with a normal platelet count was followed by complete haematological remission. 6 months later she developed a haemolytic crisis which responded to ACTH therapy. A year later she developed a fatal septicemia.

Severe haemolytic anaemia in sarcoidosis is rare and to date only 13 patients have been reported. The salient features of these together with the present case have been summarized in table I. It is apparent that hae

Table I Haemolysis

Reference	Age	Sex	Race	Lowest haemoglobin	Peak reticulocyte count, %	White cell count/μl	Platelet count/μl
CRANE and ZETLIN [7]	45	F	Negro	41%	44	15,600	150,000
STATS *et al.* [41]	8	F	Negro	43%	15	6,600	270,000
MCCORT *et al* [25]	58	M	white	55%	NR	9,900	NR
BRUSCHI and HOWE [2]	45	F	Negro	5 2 g%	22	1,900	274,000
SCHUBOTHE [38]	16	M	white	NR	incr.	NR	NR
DAVIS *et al* [8]	34	M	Negro	6 1 g%	12	6,800	204,000
LEBACQ *et al.* [22]	23	F	white	32%	23 6	10,700	NR
MICHON *et al* [26]	52	M	white	7.7 g%	NR	4,800	NR
JOHANSSON [17]	53	F	white	24%	24%	30,000	409,000

in sarcoidosis

Antibodies Coomb's test	Diagnosis confirmed by	Involvement of spleen with sarcoid granulomata	Management	Outcome
NR	lymph node bone marrow	–	splenectomy	fatal haemolytic crisis 5 days after splenectomy
NR	axillary lymph node	unknown	non-specific	NR
NR	mediastinal lymph node	–	splenectomy	recurrence of haemolytic anaemia 8 months after splenectomy. Final outcome not known
not demonstr	liver, spleen lymph node	+	splenectomy	recurrence of haemolytic anaemia 8 months after splenectomy. 5 years later patient well without treatment
direct Coomb's +	sarcoid changes in the lung	unknown	corticosteroids	remission following corticosteroid therapy; no long-term follow up
Coomb's test –	cervical mass lymph tissue	+	corticosteroids and splenectomy	haemolytic crisis 3.5 months after splenectomy; remission following corticosteroid therapy. 10 months follow-up
direct Coomb's +	spleen	+	corticosteroids	haemolytic anaemia occurred for the first time 6 months after splenectomy for a cytopenic syndrome; initial response to corticosteroid therapy but patient died from septicemia
direct and indirect Coomb's +	lymph node	unknown	corticosteroids and ACTH	remission following corticosteroid therapy. 6 months follow-up
direct Coomb's +	lungs, hilar glands	–	corticosteroids and splenectomy	no response to corticosteroids; persistence of haemolysis after splenectomy; died from cerebral [illegible] 3 months after splenectomy

Table I

Reference	Age	Sex	Race	Lowest haemoglobin	Peak reticulocyte count, %	White cell count/μl	Platelet count/μl
GARCIA *et al* [13]	31	M	white	76%	23	4,100	normal
WEST [42]	32	F	white	6 0 g%	29 8	12,100	414 000
COX and DONALD [6]	29	M	white	NR	8	11,250	normal
WYSS and MAIER [44]	35	M	white	48%	55	4,500	180 000
Present case	53	M	white	52%	12	26 000	254 000

NR = Not recorded

molysis recurred in 3 [2, 8, 25], did not remit in 3 [6, 7, 17] and developed for the first time in 2 [22 and the present case] of the 11 patients who underwent splenectomy Furthermore, only 7 of the 11 spleens examined contained sarcoid granulomata (table I), but in this context, it may be relevant that FARRER-BROWN *et al* [11] have recently stressed that meticulous care is required to detect deposits of Hodgkin's disease in excised spleens and no doubt a similar technique is required in order to reveal splenic granulomata in sarcoidosis Nevertheless, the above observations suggest that splenic involvement is not the prime factor in the genesis of haemolytic anaemia in sarcoidosis In some patients an autoimmune reaction must receive consideration It is also reasonable to suppose that reticulo-endothelial tissue elsewhere in the body may have an aetiol-

(continued)

Antibodies Coomb's test	Diagnosis confirmed by	Involvement of spleen with sarcoid granulomata	Management	Outcome
NR	lymph nodes, hypercalcemia	+	splenectomy and corticosteroids	remission following splenectomy and corticosteroid therapy. No long term follow-up
Coomb's −	mediastinal mass	+	splenectomy	remission following splenectomy, follow-up for 1.5 months
Coomb's +	supraclavicular lymph node	−	splenectomy and corticosteroids	no remission after splenectomy and corticosteroid therapy, ultimate outcome not recorded
Coomb's −	lymph nodes, lungs	+	splenectomy	remission following splenectomy, no long-term follow-up
direct Coomb's +	spleen, liver, lungs, kidneys	+	none	haemolytic anaemia occurred for the first time 1 year after splenectomy for pancytopenia, patient died in acute haemolytic crisis

ogical role in both the causation and maintenance of the haemolytic process.

The best treatment of haemolytic anaemia in sarcoidosis remains uncertain. Splenectomy has been effective in only a minority of the patients [42, 44], whereas some [illegible]

scribed by *Cox* and *Donald* [6] and *Johansson* [17]. The case described by *Crane* and *Zettel* [7] died in an acute haemolytic crisis 5 days after splenectomy and the patient reported by *Johansson* [17] who had failed to respond to splenectomy and corticosteroids died from a cerebral insult. Our patient who had undergone splenectomy for a pancytopenia died a

year later in an acute haemolytic crisis before therapy with corticosteroids or immunosuppressive drugs could be instituted

Acknowledgements We are grateful to Dr A J REYES of the Cardiovascular Unit, University of Leeds, and Dr H LEDERER of the Doncaster Royal Infirmary, for the translation of the articles

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Acta haemat 53 241-248 (1975)

Acute Myelo-Monocytic Leukaemia: A Terminal Complication of Paroxysmal Nocturnal Haemoglobinuria

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Key Words Myelo-monocytic leukaemia PNH Praeleukaemia

Abstract The authors report a case of acute myelo-monocytic leukaemia occurring in a patient, 3 years after the beginning of a typical paroxysmal nocturnal haemoglobinuria (PNH) An intermediate phase characterized by worsening of the anaemia was observed, with disappearance of the *in vitro* haemolysis tests. The kinetic studies showed a replacement of the early peripheral haemolysis by a bone marrow insufficiency with intra medullary cell death. A review of the five previous reported cases of this association show that PNH is generally a typical one so that acute leukaemia must be considered as an unfrequent but possible complication of any PNH

It is well known that paroxysmal nocturnal haemoglobinuria (PNH) may be associated to aplastic anaemia [16], refractory anaemia [1, 24], or myeloproliferative diseases such as myelofibrosis [11] The PNH syndrome, present since the beginning of these diseases, or occurring during their course is either conspicuous with clinical manifestations or limited to positive *in vitro* lysis test. Aplastic or refractory anaemia may evolve also in acute leukaemia [7, 15, 17] Consequently, DAMESHEK [5] hypothesized in 1967 a link between PNH and acute leukaemia, both conditions resulting from abnormal clones developing in these diseases, whether idiopathic or secondary to toxic or radiations This was confirmed soon after by five reported cases of acute leukaemia in patients suffering from PNH [3, 12-14, 23] In the following case, when acute leukaemia was observed the early peripheral haemolysis was replaced by an intra medullary cell death

Case Report

1st phase PNH Mr S D., a 21 year-old man, is referred in 1968 in the haematological department for bicytopenia noticed during the treatment of a sorethroat He noted for 1 year a gradual onset of pallor and weakness His past history was unremarkable, as so as the family history However, in 1966, while in the army, he attended from a ship to an atomic bomb explosion, and he was told about a 'negligible' contamination of the drinkable water

Physical examination showed nothing but skin and mucous pallor, with slight conjunctival icterus *There was no enlargement of liver, spleen, or lymph nodes* Nocturnal urins were darker than during the day, with frank haemoglobinuria

The haematological data resulted in diagnosis of PNH RBC 2 6×10⁶/mm³, Hb 7 6 g%, PCV 25%, MCV 98 μm³, MCHC 31%, reticulocytes 8 1%, WBC 4 4×10³ (PMN 2 4, lympho 1 6, mono 0.3) Platelets 74×10³ On blood smears, many polymorphonuclears with Pelger's abnormality and giant platelets were observed Bone marrow aspirate was hypercellular, with gross erythroid hyperplasia, some inter nuclear bridges between erythroblasts and a slight histio-monocytic infiltration (proerythroblasts 3%, erythroblasts basophilic 10% polychromatophilic 20%, acidophilic 13%, myeloblasts 2%, promyelocytes 2.5%, myelocytes 2% metamyelocytes 6% poly neutro 13%, eosino 1% monocytes 4.5%, round cells 19%) Serum iron 170 μg%, TIBC 305 μg%, serum total bilirubin 1 9 mg%, Coombs test negative Haemosiderinuria ++ after Perls' staining, with 24 hour urinary iron excretion between 3 and 6 mg The Ham Dacie test was positive, as so as *in vitro* lysis tests with thrombin or sucrose The red cell acetyl-cholinesterase was slightly lowered (0 44 U, normal 0.5–1) The leucocyte alkaline phosphatase was absent Iron kinetics showed with ^{59}Fe the typical pattern of PNH plasma clearance was rapid with important fixation in sacrum, the red cell incorporation began early, at the third day, and increased to 50% with the characteristic curve break indicating an early peripheral haemolysis

During 2 years, the course of the disease was characterized by haemoglobinuric crisis occurring at various moments and requiring transfusions of washed red cells, approximately each 2 weeks, then each month No improvement was observed after corticoids androgens or anti vitamin K

2nd phase PNH + excessive myeloblastosis At the end of 1970, the patient suffered from herpes zoster and corticotherapy was stopped Then the frequency of haemolytic crisis increased. The anaemia was more severe and the transfusions more difficult since the appearance of anti K, E and platelet antibodies Hepatosplenomegaly developed

At this time progressive modifications of the haematological data were observed the reticulocyte count decreased to 138 then 51×10³/mm³, the WBC to 2 8 then 1.3×10³/mm³ with marked neutropenia, and the platelets to 46×10³/mm³ There were rare immature circulating cells Haemoglobinuria and haemosiderinuria were less pronounced, and the *in vitro* lysis tests were less positive (4% then <1% lysis with Ham test, and 20% then <2% with sucrose) The red cell acetyl cholinesterase increased to 0 72 U The marrow aspirate was still hypercellular but with less erythroblasts (29%) and with an excess of myeloblasts (11.5 then 15.5%) and of mono-

cytes (7%). The myeloblasts, with frequent monocytoid features, had the cytochemical features of the granulocyte series (peroxydase +, Sudan black B +) and rare but unmistakable Auer bodies were observed. The study of the karyotype revealed no chromosomal abnormality. The search for some erythrocytic abnormalities was negative: there was no positive PAS staining of erythroblasts, nor hypersideroblastosis. The activities of G-6-PD, 6-PGD, hexokinase and pyruvate kinase were normal. Only a moderate increase of HbF to 3% was observed.

Many differences appeared at a second ferrokinetic studies: the erythrocytic incorporation of ^{59}Fe was strongly reduced (maximum 10%), this bone marrow deficiency preventing the identification of a persistant early peripheral haemolysis. Insufficient erythropoiesis was also evidenced with a marked contrast between a still satisfactory bone fixation and a slow and incomplete release of ^{59}Fe. This disorder was confirmed by autoradiography study carried out after *in vitro* labelling of bone marrow erythroblasts with ^{3}H thymidine: the construction of a model according to the method developed by NAJEAN *et al.* [18] was compatible with a significant intramedullary haemolysis.

At the end of this period, the anaemia worsened. An attempt of chemotherapy with 6-mercaptopurine and cytosine-arabinoside resulted in severe bone marrow failure, complicated with *Klebsiella septicemia*, followed by a return to the previous haematological status with excessive myeloblastosis.

3rd phase: acute myelo-monocytic leukaemia. This condition appeared in November 1971, characterized by hyperleucocytosis (WBC $13.4 \times 10^3/mm^3$, then 86×10^3 2 months later) with blood myeloblastosis. The marrow was markedly hypercellular and contained 55.5% leucoblasts of the myelo-monocytic type. There were still many erythroblasts (30.5%) with arrest of their maturation at a very early stage. Numerous megakaryocytes were also observed, contrasting with a peripheral thrombopenia ($11 \times 10^3/mm^3$). The myelo-monocytic type of this acute leukaemia was confirmed by the high levels of lysozyme in serum (42.5 $\mu g/ml$) and urine (47 mg/l). At this stage the clinical and biological signs of PNH had completely disappeared with negative *in vitro* lysis tests.

The course of the disease was very short: a marked enlargement of liver (20 cm high), spleen (7 cm beyond the costal margin)) and lymph nodes was observed. The patient suffered from pruritus, buccal and anal ulcerations. He died on February 1972 from septicemia with pulmonary manifestations, renal failure and ileus. The autopsy showed, aside the lesions related to septicemia and diffuse haemorrhagic syndrome, a leukaemic infiltration of bone marrow, liver, spleen, as well as cutaneous, muscular and gastric areas – this last infiltration presenting as a necrotic and haemorrhagic pseudo-tumour (Prof. J. DIEBOLD).

Discussion

With our patient, there are now six reported cases of PNH terminating in acute leukaemia (table I). Although these observations support DAMESHEK's [6] opinion that any PNH is a potentially pre-leukaemic condition,

Table 1 Association PNH acute leukaemia

Reference	Age years	Sex	Delay, months				PNH		Type of acute leukaemia	Disappearance PNH when leukaemia occurs
			first sign diagnosis PNH	diagnosis PNH acute leukaemia	acute leukaemia-death	intermediate pre-leukaemic phase	haemoglobinuria	treatment		
Kaufmann *et al* [14]	58	M	24	30	6	24	+	androgens + iron	myeloblast + myelofibrosis	
Holden and Lichtman [12]	37	F	14	72	few	12	+	corticoids + iron	erythro-monocytic	+
Jenkins and Hartmann [13]	31	F	36	8	7	3	+	corticoids	myelobl ?	+
Carmel *et al* [3]	32	M	1	4	complete remission	1	0	vitamins	erythro-myelobl	+
Tsevrenis *et al* [25]	50	M	36	36	4	0	+	corticoids, folic acid	*erythro L*	
Present case	21	M	12	47	3	9	+	androgens + corticoids	erythro-myelo-monocytic	+

it is probably a rare occurrence that has not been mentioned in important series and reviews until 1967 [4]. The clinical and biological features of PNH are characteristic in 5 out of 6 cases with paroxysmal haemoglobinuria, haemosiderinuria, positive *in vitro* lysis tests with acid, sucrose or thrombine, decrease of erythrocytic acetyl-cholinesterase [13] and of leucocytic alkaline phosphatase [14, 25]. Usual complications may be observed such as allo-immunisation [3, 13] and thrombo-embolism. In only one single observation reported by CARMEL *et al.* [3] PNH was restricted to *in vitro* lysis test without haemoglobinuria during a pre-leukaemic syndrome with circulating myeloblasts since the beginning, the acute leukaemia supervening rapidly. The possibility of such positive tests and/or decrease of erythrocytic acetyl cholinesterase activity during common leukaemia or myeloproliferative diseases have already been emphasized [9].

In the five other cases, acute leukaemia occurred after a prolonged course of typical PNH (from 4 to 7 years) and was characterized by worsening of anaemia with decrease of reticulocytes, hepatosplenomegaly and lymph node enlargement [12, 13, 25]. The decrease of neutrophils and platelet led to infections and haemorrhagic complications and patients died within 3 to 7 months after unsuccessful chemotherapy. Autopsy conducted in the case of KAUFMANN *et al.* [14] and in our case showed the usual features of acute leukaemia with massive visceral infiltration. Before acute leukaemia became evident, an intermediate phase was observed, lasting from 1 to 24 months and characterized by progressive bone marrow failure [3, 12–14] and hepatosplenic enlargement [13]. The haematological examinations showed some abnormalities of pre-leukaemic signification: hypochromasia [3, 25], acquired Pelger's abnormality [13], monocytosis [13], circulating immature cells such as erythroblasts, myelocytes [14, 25] and even some myeloblasts [3, 12, 13]. Bone marrow aspirates were difficult to get [12] because of myelofibrosis [14] and showed a relative excess of myeloblasts [12, 13]. Chromosomal abnormalities could have a prognostic value in view of their significance in the pre-leukaemic conditions [20] and of their possible existence in PNH [26]. In fact only two cases have been studied: aneuploidy was found by CARMEL *et al.* [3] but there was no chromosomal abnormality in our case. At last the existence of possible leukaemogenous etiologic factors may be interesting but such factors were not reported, except in our patient who undergone perhaps a contamination during an atomic explosion.

The acute leukaemia reported after PNH included frequently erythroblastic proliferation with megaloblastosis [3, 12, 25] and in our case

as well as in HOLDEN's one [12], was of the myelo-monocytic type It is interesting to point out that the acute malignancy evolving after acquired erythrocytic diseases such as refractory anaemia [2, 15, 27] or polycythaemia vera [23] are frequently of the erythroleukaemic type, the possible association of a monocytic proliferation [8] deserves the name of erythromonocytic leukaemia Myelo-monocytic leukaemia, which involves the myeloid stem cell with abnormalities of all three myeloid lines [22], is the condition which exhibits the more frequent and multiple erythrocytic defects [28]

A decrease or disappearance of clinical and biological symptoms of PNH when acute leukaemia occured has been noticed in three other cases [3, 12, 13] The kinetic study carried out in our patient showed, however, that it is not a simple regression of a qualitative defect indicating the disappearance of the abnormal clone Out study shows, indeed, that a severe bone marrow insufficiency makes it difficult to find again an early peripheral haemolysis, taking into account the very low erythrocytic incorporation of ^{59}Fe Moreover, inefficient erythropoiesis appeared, and the kinetic model constructed after ^{3}H thymidine autoradiography suggests an haemolysis at a more primitive, intra-medullary stage In fact numerous proerythroblasts and basophilic erythroblasts persisted in the bone marrow, with 'maturation arrest' It can be assumed, therefore, that the characteristic abnormality of PNH which causes *in vivo* haemolysis, far from disappearing, involves during leukaemic transformation the most primitive stages of erythrocytic lines, thus inducing intra medullary cell death This is however only an hypothesis the cause of *in vivo* haemolysis in PNH remains unclear, and an intra medullary lysis associated with quantitative deficiency is usually found in malignant conditions, especially in acute leukaemia [19], or at a late phase of polycythaemia vera [21]

Acknowledgment We are grateful to Prof Y NAJEAN and Dr C. DRESCH who performed the iron kinetic studies and autoradiography with ^{3}H thymidine

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Acta haemat. 53: 249-255 (1975)

Anti-Heparin Activity of a Macroglobulin from a Patient with Breast Adenocarcinoma

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Key Words. Antiheparins · Bleeding disorders · Macroglobulin · Paraproteinemia · Platelet aggregation

Abstract. We describe a case of IgM paraproteinemia, whose macroglobulin reacted anomalously with heparin. Anti-heparin activity of the paraprotein was suggested by insensibility of the patient's plasma to heparin in heparin-thrombin clotting time. Moreover, the presence of heparin in the medium markedly reduced the inhibitory effect of the paraprotein on platelet aggregation by ADP.

Dysproteinemic syndromes are frequently associated with coagulation anomalies, which may result in a bleeding tendency [5, 6, 8, 10] or, sometimes, in thrombosis [1, 11].

Recently we observed a case of IgM paraproteinemia, in which two opposite anomalies were present: the patient had a bleeding tendency and, at the same time, an insensibility to heparin due to an anomalous interaction between the paraprotein and heparin.

Case Report

A. M., a housewife aged 5[illegible], was admitted to our haematological division in November 1971 because of symptomatology characterized by deep asthenia, thoracic pains, paresthesias, auricular buzzings and headache. Blood analysis showed slight anemia (Hb 10 g/dl) and a clearly altered electrophoretic pattern. Electrophoresis on cellulose acetate detected the presence of a large peak in the γ region (total proteins 11.2 g/dl, of γ fraction 4[illegible]%), which by immunoelectrophoresis was shown to be formed by an IgM k type. After ultracentrifugation several different components with high sedimentation constants were noted, the most common component

having a sedimentation constant of 28.2 S. Bence Jones protein was not found in the urine. Abnormal data for haemostasis are reported in the results.

Since a bleeding tendency was present, several plasmaphereses were performed. The electrophoretic pattern and the haemostasis indexes clearly improved. The patient was discharged in good general condition. About 1 year later, during a periodical examination, a large, hard irregular nodule adherent to the superficial and deep layers was detected in the right breast. The axillary lymph glands were not palpable. Liver and spleen were normal. Several plasmaphereses were performed to prepare the patient for surgery. A right mastectomy was carried out with emptying of the corresponding axillary nodes. From histological examination of the mammary nodule a diagnose of breast adenocarcinoma was made. The post-operative period was good and the patient was discharged a month after the operation in tolerable general condition.

Materials and Methods

Heparin (Liquemin, Roche) was diluted in saline; a stock solution (10 IU/ml) was kept at −20 °C in small aliquots until use; serial dilutions (from 0.25 to 10 IU/ml) were prepared just before the test.

Thrombin (Topostasine, Roche) was diluted in saline; a stock solution (30 NIH units/ml) was kept at −20 °C in small aliquots until use; these were thawed just before the test, diluted in saline to 10 NIH units/ml and kept on ice during the test.

Adenosine 5-diphosphate (ADP, Biochemia), dissolved in buffered saline pH 7.4, was used at final concentrations of 8×10^{-6} or 8×10^{-7} M.

Thrombofax (TBX, Orto) was used as stock solution.

Preparation of platelet rich (PRP), platelet poor plasma (PPP) and serum. Blood samples from the patient and normal subjects were added to citrate (one part of 3.8% trisodium citrate to 9 parts of blood) or heparin (0.2 ml heparin 100 IU/ml to 9.8 ml blood) and centrifuged at 400 g for 10 min to obtain the PRP and at 2,000 g for 20 min to obtain the PPP. All equipment for handling blood and plasma was plastic or siliconized. Serum from the patient's blood was obtained after 4 h incubation at 37 °C in glass tubes and centrifugation at 2,000 g for 20 min.

Gel filtered platelets (GFP) were obtained by the method of TANGEN *et al.* [12].

Coagulation studies. The following standard assays were used in the coagulation studies: standardized bleeding time [MIELKE], clotting time [LEE WHITE], platelet count [FEISSLY LÜDIN], prothrombin time [QUICK], thrombin time [BRIGGS], partial thromboplastin time [MACFARLANE], plasma recalcification time [HOWELL], thromboelastogram [HARTERT], clot retraction [FONIO], coagulation factor VIII [BRIGGS], serum fibrin(ogen) derivative products (FDP) [MERKSEY]. Fibrinogen was determined by a method of heat precipitation [RECALCATI].

Heparin thrombin clotting time was performed at 37 °C by addition of 0.05 ml of various concentrations of heparin and 0.1 ml thrombin (10 NIH units/ml) to 0.25 ml PPP, according to the method of HARADA and ZUCKER [3].

Platelet aggregometry. The changes in optical density of both PRP and GFP induced by ADP and TBX were measured in an EEL 169 platelet aggregation meter

Table I. Routine coagulation studies and effect of plasmaphereses on coagulation tests

	Before plasmaphereses	After plasmaphereses¹	Normal
Bleeding time, min	8	3	<4
Platelets × 10³/µl	150	200	250–350
Prothrombin time, sec	14.5	12.5	12–14
Thrombin time, sec	24	17	17–24
Partial thromboplastin time, sec	60	42	35–40
Howell time, sec	240	150	80–150
Plasma clot retraction	*	***	****
Fibrinogen, mg/100 ml	310	350	200–400
Factor VIII %	90	90	95
Thromboelastogram			
r	26	–	7–10
k	31		6–8
ma	30		50–65
Serum FDP	absent	absent	absent

¹ 3.0 litres of plasma removed over a 7-day period.

(Evans Electroselenium Ltd.) connected to a pen recorder (Servogor 1 near logarithmic integrating recorder) to permit the automatic registration of variations in the transmitted light. The sensitivity was adjusted so that PPP or buffer (in the case of GFP) was set to 0% . Reagents were added GFP) was set close to 100% and PRP or GFP was set to 0%. Reagents were added to the cuvette of the aggregometer as follows: 0.8 ml PRP or GFP, 0.2 ml saline or PPP and 0.2 ml ADP or TBX.

Results

Coagulation and fibrinolysis studies. The results are reported in table 1. Prolonged bleeding and Howell times, a deficient plasma clot retraction and an altered thromboelastogram were observed before plasmaphereses.

Anti-heparin activity of macromolecule. After addition of heparin to the patient's plasma or serum a dense precipitate appeared. The heparin thrombin clotting time was not prolonged by increasing concentrations of heparin added to the patient's citrated PPP until a concentration of 10 IU/ml (fig. 1). Anti-heparin activity also was present in the patient's serum.

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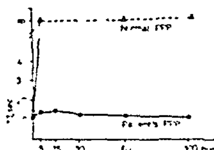


Fig. 3. Thrombin time (TT) of normal and the patient's PPP after intravenous injection of heparin (50 mg)

Table II. Platelet aggregation by ADP (8×10^{-6} M) and TBN of normal and the patient's PRP

	Platelet aggregation, %	
	patient	normal
ADP	50	90
TBN	45	90

Table III. Effect of addition of the patient's citrated PPP (C-PPP), the patient's heparinized PPP (H-PPP) and normal citrated PPP on the aggregation both of normal and the patient's gel filtrated platelets (GFP)

	Platelet aggregation by ADP (8×10^{-6} M), %		
	patient's C-PPP	patient's H-PPP	normal C-PPP
Patient's GFP	0	30	50
Normal GFP	20	80	90

whereas 90% was observed with normal PRP (table II). Moreover, the addition of the patient's citrated PPP to gel filtered platelets both from the patient and from a normal subject provoked a marked inhibition of platelet aggregation by ADP (8×10^{-6} M). On the other hand, the addition of the patient's heparinized PPP to the platelets had only a slight inhibitory effect on platelet aggregation by ADP (table III).

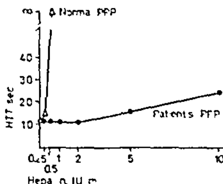


Fig 1 Heparin thrombin clotting time (HTT) of normal and the patient's PPP

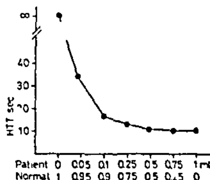


Fig 2 Heparin thrombin clotting time (HTT) of mixtures of normal and the patient's plasma

When increasing concentrations of the patient's plasma were added to normal plasma the heparin thrombin clotting time of the mixture was decreased (fig 2) Even very low concentrations of the patient's PPP (5%) reduced the clotting time to a measurable value

The effect of heparin *in vivo* was studied by injecting a standard dose (50 mg) intravenously into the patient and into a normal subject of the same age and sex Thrombin time was determined at various times after injection (fig 3) Although the thrombin time of the normal PPP was greater than 1 min within 5 min after heparin injection the thrombin time of the patient's PPP remained about 13 sec

Platelet aggregation in the presence of the paraprotein When ADP ($8 \times 10^{-?}$ M) or TBX were tested for platelet aggregation against the patient's PRP only 50% aggregation was registered in the aggregometer

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Discussion

The blood of the patient contained a paraprotein which reacted anomalously with heparin, forming a precipitate visible to the naked eye The protein was shown by immunological analysis to be an IgM, λ type, present in the plasma as a complex Studies on the physical and chemical structure of the complex are in progress The paraprotein was responsible for the haemostatic changes (bleeding time and clot retraction), which improved after repeated plasmaphereses

Furthermore, from studies with gel filtered platelets, it was shown that the paraprotein interfered with platelet aggregation induced by ADP and Thrombofax [2], probably masking the specific receptors for aggregating agents The presence of heparin in the reaction mixture markedly reduced the inhibitory effect of the paraprotein Anti-heparin activity of the paraprotein was suggested by insensibility of the patient's plasma to heparin in heparin-thrombin clotting time It was confirmed by demonstrating that the patient's plasma shortened the heparin-thrombin clotting time of normal plasma, even at very low concentrations Moreover, the intravenous injection of a high dose of heparin did not prolong the thrombin time

In 1960, MILLER [7] described an IgM macroglobulin which was able to precipitate heparin *in vitro* GLUECK *et al* [4] reported a patient who was a carrier of a paraprotein IgG, λ type, and who had three thrombotic events that disappeared after administration of extremely high doses of heparin It is probable that in the case described by GLUECK *et al* the anomalous globulin exerted a 'procoagulant' action We cannot exclude an analogous action for the paraprotein of our patient It may be significant that in our patient the clinical symptoms of bleeding were not remarkable, although several abnormalities of coagulation and platelet function were present

The nature of the interaction between heparin and paraprotein is not clear Immunological binding between the complex and heparin is a possible explanation, but non specific affinity cannot be excluded Studies on the chemical structure and the nature of the interaction between heparin and macroglobulin are in progress

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Acta haemat. 53: 257–276 (1975)

Hairy Cell Leukemia ('Leukemic Reticuloendotheliosis'), Reticulosarcoma, and Monocytic Leukemia

Cytochemical and Ultrastructural Investigations

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Key Words: Cytochemistry · Electron microscopy · Hairy cell leukemia · Monocytic leukemia · Reticulosarcoma · Reticulosis

Abstract. Cytochemical and electron-microscopic studies have been carried out on leukemic monocytes and 'hairy cells' (HC), reticulosarcoma (RS) cells and cells of cases of 'reticulosis' and 'reticulosarcoma cell leukemia'. Additional investigations included quantitative determinations of the urinary lysozyme excretion, skin window studies, testing of the phagocytosis of ferritin by HC, and labeling of the Fc receptors on HC at the ultrastructural level. Clear evidences against any cytological relationship among leukemic HC and monocytes have been provided. Further results argued also against the frequently stressed relationship among leukemic monocytes and RS cells. Cases of 'RS cell leukemia' and 'reticulosis' had to be reclassified as lymphosarcoma cell leukemia, acute lymphatic and myeloblastic leukemias. Besides distinct ultrastructural differences among HC, RS cells, and lymphocytes, mainly gradual differences have been noted using cytochemical methods and by evaluating the phagocytosis of ferritin particles. A further common trait of HC, RS cells, and B lymphocytes seems to be the presence of surface Fc receptors. A more precise classification instead of the diagnoses 'reticulosarcoma' and 'reticulosarcoma cell leukemia' is required, and the use of the term 'hairy cell' leukemia is suggested instead of the misleading term 'leukemic reticuloendotheliosis'.

The origin of the blood monocytes from the reticuloendothelial system (RES) was first proposed by Schilling and subsequently was widely accepted [for literature see 33, 64]. Accordingly, monocytic leukemia continued until quite recently to be defined as the merely leukemic manifestation of a generalized malignant process of the RES – as opposed to the localized tumorous reticulosarcoma. Development of leukemia following a primary 'reticulo-

MATHÉ, G SCHWARZENBERG L. and POUILLART, P Nomenclature, Methodology, and Results of Clinical Trials in Acute Leukemias; in RENTCHNICK P (ed) Recent Results in Cancer Research, Vol 43 Springer Berlin 1973 X + 168 pp fig 79, tab 64 DM 58.–/US $ 23 80

Diese Publikation basiert auf den Mitteilungen die an einem Internationalen Workshop am CNRS in Paris im Juni 1972 präsentiert wurden Bei der akuten Lymphoblastenleukämie (ALL) liegt das Schwergewicht eindeutig auf der Unterteilung in 4 zytologische Typen welche sich mit konventioneller Färbung unterscheiden lassen Dass diese neue Klassierung grosse diagnostische therapeutische und prognostische Bedeutung hat wird vor allem am Patientengut aus Villejuif dokumentiert Zytochemie und Elektronenmikroskopie leukämischer Zellen werden anhand der Daten aus verschiedenen Zentren neu auf ihre Aussagekraft geprüft Vor allem bei der Zytochemie ist auffallend wie gross die Divergenzen der Meinungen sind Dies kommt einerseits zustande durch methodische Unterschiede und andererseits durch die subjektive Interpretation von Positivität bzw Negativität Die Akten über den Wert der Zytochemie in der Diagnostik der akuten Leukämien konnten also auch bei diesem Workshop durchaus nicht geschlossen werden Die therapeutischen Trials bei ALL an verschiedenen Zentren zeigen erfreulich gute Resultate, und neue Wege scheinen sich anzubahnen um die Prognose noch weiter verbessern zu können Bei der akuten myeloischen Leukämie (AML) sieht es trotz höherer Remissionsraten noch düster aus Vor allem wird man pessimistisch gestimmt durch die kooperative Studie der europäischen Gruppe (EORTC) Im ganzen ist das Buch für Hämatologen und Onkologen sehr stimulierend und gibt einen guten Überblick über den heutigen Stand in der Diagnostik und Therapie der akuten Leukämien

B SPECK *Basel*

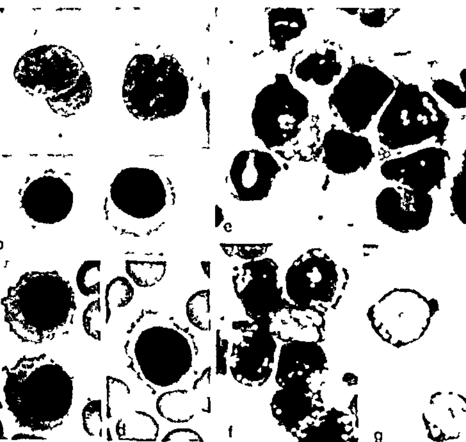


Fig. 1. a Typical leukemic monocytes. b Typical hairy cells; the nuclei are [illegible] [illegible] The cytoplasmic processes [illegible] may vary in size and frequency as well as in the superficial [illegible] are [illegible] [illegible] Reticulosarcoma (RS) of the bone (RS cell) [illegible] of a pleural effusion. Note the [illegible] of the nuclei in the different cells. [illegible] RS cells of a lymph node [illegible] [illegible]



sarcoma' or 'histioblastic lymphoma' and occurrence of tumors in monocytic leukemia were felt to corroborate SCHILLING's opinion. Confusion arose as BOURONCLE *et al.* [7] termed 'leukemic reticuloendotheliosis' an obviously new recognized nosological entity, referring to EWALD [13], who in 1923 – using this term – described what he thought to be a leukemic manifestation of a malignant process involving primitive cells of the RES.

However, recent investigations led to a thorough characterization of the normal blood monocytes so far as their myelogenous origin [16, 17, 33, 53, 57], their kinetics [18, 39], their ultrastructure [6, 19, 26, 44, 65] and cytochemistry [33, 44, 55, 67], their functional capacities [16, 55], and their immunological membrane properties [25] are concerned. These peculiar features unequivocally distinguish monocytes from other hematopoietic or blood cells. Furthermore, monocytic leukemia has been studied with the same techniques, and their close relationship to normal monocytes and their physiological precursors has been proved [4, 15, 25, 27, 33, 52, 56].

We studied the cytochemistry, ultrastructure, and functional capacities of the leukemic cells in patients with 'hairy cell' and monocytic leukemia as well as with 'reticulosarcoma' and 'reticulosarcoma cell leukemia' (RC-leukemia). The results and conclusions drawn from these comparative studies are presented.

Material and Methods

Blood and bone marrow smears were studied in the following cases:

17 patients with monocytic leukemia. The diagnosis of monocytic leukemia was made following to previously outlined criteria [52, 56]. According to these, 2 cases were defined as 'immature' monocytic leukemia. 15 patients exhibited the criteria of 'mature' monocytic leukemia (fig. 1a).

One patient, whose histological examination of the bone marrow biopsy prompted the diagnosis of 'reticulosis' (presence of considerable argyrophilic reticulin fibers). However, according to the cytochemical and ultrastructural criteria, this case was classified as atypical 'monomyelocytic' leukemia [58] (case 3).

10 patients suffering from 'hairy cell' leukemia (HC-leukemia) (fig. 1b–d).

4 patients with the first diagnosis of 'reticulosarcoma' who subsequently developed frank leukemia (RC-leukemia, fig. 1e). Smears and imprints of 'reticulosarcoma' cells were studied as follows: Imprints of 4 lymph nodes in which the histological diagnosis of 'reticulosarcoma' had been made. Smears of pleural effusion of a patient cited in the aforementioned group in which conspicuous 'reticulosarcoma' (RS) cells were present in the pleural fluid (case 9, fig. 1f). Smears from bone marrow aspiration of a patient (case 10) suffering from a 'reticulosarcoma of the bone' with infiltration of the bone marrow, but without neoplastic cells in the peripheral blood.

Cytochemical stainings were performed in all smears and imprints listed above. The

Table I

Case No	Cytological type of leukemia or tumor	Number of cases	POX	SuB	Chloracylesterase	AcP−L+-tartrate	AcP+L+-tartrate
1	monocytic [1] mature	15	(+)		(+)	++	
			−	+	−	−	(+)
			+		+	+++	
2	immature	2	(+)		(+)	+	
			−	+	−	−	(+)
			+		+	+++	
3	monomyelocytic [1]	1	+	+	(+)	+	
			−	−	−	−	(+)
			+++	+++	++	+++	
4	lymphosarcoma cell [1]	2					
			−	−	−	+	n d
5	undifferentiated [1]	1				(+)	
			−	−	−	−	n d
						+	
6	myeloblastic [1]	1	(+)		−	(+)	
			−	+	−	−	n d
			+		+	+	
7	'hairy cell' [1]	10		−		+	+
			−	−	−	−	−
				(+)		+++	++
8	'reticulosarcoma' lymph node imprints	4		−[1]		−	−
			−	−	−	−	−
				(+)		+	+
9	smears from pleural effusion	1		−[1]		−	−
			−	−	−	−	−
				(+)		+	(+)
10	bone marrow aspirate	1		−[1]		(+)	−
			−	−	−	−	−
				(+)		++	+

− = Negative (+) = uncertain positive staining, + = weak positive staining, ++ = moderate positive staining, +++ = strong positive staining n d = Not done

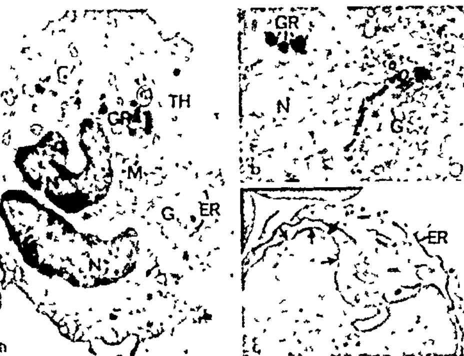


Fig. 3. a Leukemic monocyte. Nucleus (N), short strands of ergastoplasm (ER), Golgi area (G), mitochondria (M), small granules (GR). A thrombocyte is phagocytized by the cell (TH). × 11,300. *b* Leukemic monocyte. Deposits of lead indicating the activity of acid phosphatase are located in the Golgi cisternae (G) and in some granules (GR). (The thin section was not stained with lead hydroxide or uranyl acetate.) × 25,000. *c* Leukemic promonocyte. Activity of peroxidase confined to perinuclear space (at cross), ergastoplasm (ER), and some granules. × 9,400.

Activity of acid phosphatase can be demonstrated in nearly all leukemic monocytes. It is localized in the perinuclear space, in the cisternae of the ergastoplasm and of the Golgi field and in some granules (fig. 3b). Activity of POX is less conspicuous. In more immature monocytes, perinuclear space, ergastoplasm, and some granules may show this enzyme activity (fig. 3c); in mature cells only a few granules may be stained.

HC are moderately large and are characterized by conspicuous small cytoplasmic protrusions (fig. 4a). The nuclei are oval, horseshoe-shaped, rounded or kidney-shaped with occasional deep indentations. The heterochromatin

staining of the HC cytoplasm with SuB is not due to peroxidatic activity as it is in the azurophilic granules of the neutrophils and monocytes [51] but probably indicates presence of lipidic inclusions. PAS staining also is inconstant and does not show the typical granular pattern present in chronic and acute lymphatic leukemia.

The cytochemical pattern of RS cells obtained from lymph nodes includes absent or weak staining for AcP, r-N-AS-E (fig. 2c) and moderate or weak staining for α-N-E. AcP was inhibited by L+-tartrate. Cytoplasmic vacuoles present in some of these cells show a weak peripheral staining with SuB probably indicating storage of lipids. The pyroninophilia of the cytoplasm is usually prominent (fig. 1g). PAS staining yielded different results, varying from virtually absent to strong positive granular staining. Actually the same cytochemical pattern is observed in the cells of pleural effusions and – with the exception of strong granular PAS positivity and a marked activity of AcP – in the cells infiltrating the bone marrow in the case of RS of the bone.

Electron microscopy. Mature leukemic monocytes are rather large cells with frequent finger-like cytoplasmic protrusions which, however, differ in shape and number from the 'villi' of the HC (fig. 3a). The monocyte nuclei *are large, irregular, frequently lobulated. Nuclear pockets are rather fre*quent. The chromatin is evenly distributed and is condensed in a small rim along the nuclear membrane. Mitochondria may be numerous and large. *Profiles of ergastoplasm are well developed, usually short.* The *Golgi* apparatus is large, and conspicuous vesicles and vacuoles are present. Besides some large granules, 0.5–0.8 μm in diameter, most cytoplasmic granules are distinctly smaller, 0.1–0.2 μm in diameter, electron dense, occasionally comma shaped. Bundles of fibrils occur as well as phagocytosis of degenerated thrombocytes and erythrocytes [15, 27].

The immature leukemic monocytes differ in so far as their nuclei are less polymorphous – usually rounded or kidney-shaped – and occasionally deep *clefts are observed. The ribosomes usually are numerous and arranged to* polysomes, ergastoplasm and Golgi apparatus are not as conspicuous as in the mature monocytes, and granules are less frequent [27, 52].

Atypical 'monomyelocytic' leukemia is characterized by large cells with a few cytoplasmic protrusions. The nuclei are large and resemble those of the monocytes. The cytoplasm appears rather dense. It contains long profiles of ergastoplasm and a well developed Golgi apparatus. Mitochondria are large and show a dense matrix with electron dense granules. The cytoplasmic granules are of different *types*, *small*, as those in the monocytes, or resembling neutrophilic granules. Rare Auer bodies could also be detected [27, 58].

is irregularly arranged along the nuclear envelope, but clumps are also distributed throughout the nuclei, corresponding to the reticular appearance of the heterochromatin following May-Grünwald-Giemsa stain and in the phase contrast microscope. Mitochondria are frequent and occasionally rather large. Some electron-dense, osmophilic lysosomes arise from the Golgi apparatus (fig. 4c). They show positive staining for AcP (fig. 4f). Some short profiles of ergastoplasm are present in these cells. Free ribosomes are moderately frequent, occasionally they are arranged to polysomes [14, 32, 49, 60]. Uropods with prominent pinocytic activity are rather frequent; if they occur, the villi are mainly localized on them. Lamellar inclusion bodies [32] were developed only incompletely (fig. 4d). We had observed a case of chronic lymphocytic leukemia with similar structures. Activity of POX never was observed in HC at the ultrastructural level.

Membrane-bound immunoglobulins were investigated in 2 patients by electron microscopy. In 5–30% of HC either IgG, IgA or IgM were detected. In most cases the POX labelled immunoglobulins were constricted to areas of microvilli formation (fig. 4a), they often were deposited in a system of small tubules and vesicles near the cell surface (fig. 4b).

RS cells are moderately large. They show few and small cytoplasmic projections when studied in lymph node sections. Nuclei are large and contain an evenly distributed chromatin and few nucleoli. Ribosomes are usually numerous and mostly arranged to polysomes, whereas the rough endoplasmatic reticulum is poorly developed and also the Golgi apparatus is not prominent and only a few lysosomal structures are present. Mitochondria are usually moderate in size and number. A few vesicles are present. Occasionally the cells may appear more mature showing distinct vacuolar structures and less ribosomes [43].

Skin window investigations. Monocytic leukemia. As already shown in previous publications, conspicuous migration of leukemic cells into the exudate occurred (fig. 5a). Consequently, these cells transformed into macrophages showing the same qualitative changes of the cytochemical pattern as in normal monocytes [55].

In the 'monomyelocytic' leukemia, the leukemic cells neither migrated specifically into the exudates nor did the few leukemic cells present amidst the other accumulated nucleated cells transform into macrophages [55].

HC-leukemia. 1 of the 2 patients studied by skin window technique contained about 70 000 HC/mm³ in the peripheral blood. In this case a few small clusters of HC were present in the exudates suggesting some motility and – perhaps – chemotaxis. However, the number of HC in the exudates never

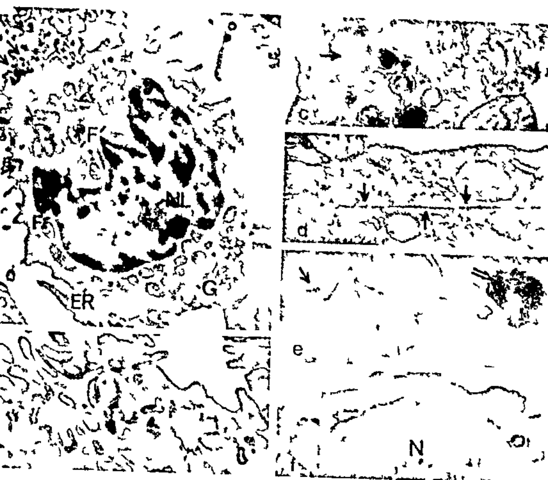


Fig 4 a Hairy cell Prom nent nucleolus (NL) Bundles of f brils along the nuclear periphery (F) Short strands of ergastoplasm (ER) pron nent Golgi zone (G) and many small vacuoles and ves cles at the periphery of the cell Membrane-bound immunoglobul ns labelled by perox dase are constr cted mostly to the area of m crovill s (arrows) × 8 600 *b* Perox dase-labelled immunoglobul ns located in small tubules and ves cles × ?? 700 *c* Small granules somet mes enclosed by a double membrane and larger depos ts of an electron-dense mater al (arrow) small ves les and tubules × 33,200 *d* Lamellar inclus on body (arrows) × ?8 300 *e* Uptake of ferrit n by a ha ry cell Ferr t n in small ves cles (arrow) and depos ted together w th an electron-dense mater al (double arrow) × 37 500 *f* Act v ty of acid phosphatase located in the perinuclear space in c terns of ergastoplasm and in small granules × 19 000

the same percentage of lymphocytes and HC take up ferritin which is found to be localized in vesicles and vacuoles; by contrast, in HC after 30 min of incubation the ferritin is additionally localized in secondary lysosomes.

LYS determination in urine. In mature monocytic leukemia the urinary excretion of LYS ranged between 90 and 700 µg/ml if leukemia was actually present. In remissions the excretion was markedly reduced [4]. In the case of 'monomyelocytic' leukemia, urinary LYS levels were normal or weakly increased. LYS excretion was normal or reduced in the patients with HC-leukemia independently of the degree of leukemia.

NBT test. No reduction of NBT and no phagocytosis of latex particles occurred in the HC.

Discussion

Relationship of leukemic HC to monocytes and monocytic leukemia. Only few authors considered a close relationship among the HC and the monocytes [40, 61, 65] and quoted the observed differences in the cytochemical patterns as merely gradual differences [40, 65]. However, recent cytochemical investigations which are confirmed and extended by our present results reveal true qualitative differences (table I) – at least as far as the most characteristic enzymes in these cells are concerned: α-N AS-E, NA and LYS as the enzymes peculiar for monocytes, and the tartrate resistant isoenzyme 5 of the leukocytic AcPs as the enzyme peculiar for HC [31, 69].

All investigators agree on the differences existing among monocytes and HC at the ultrastructural level [9, 14, 32, 43, 60]. As far as the function is concerned, no consensus exists on the phagocytic ability of HC. Relevant uptake of latex particles did not occur in our hands, and herein we are in accordance with the findings reported by SCHREK and DONNELLY [61] and YAM *et al.* [65]. RUBINS *et al.* [49] and HAAK *et al.* [22] could not detect phagocytosis of bacteria by the HC. FLANDRIN *et al.* [14] noted an uptake of latex and DANIEL and FLANDRIN [11] were able to confirm these findings with the electron microscope. KATAYAMA *et al.* [32] observed an in vivo phagocytosed erythrocyte within an HC, which, however, had no typical villi on its surface. In order to prove the phagocytic or pinocytic activity, we studied the uptake of ferritin particles with the electron microscope. HC showed a weak intracellular accumulation of ferritin, which was in the same range as that of lymphocytes and was markedly less than that seen in monocytes. However, in contrast to lymphocytes, after 30 min of incubation the HC contained ferritin also in secondary lysosomes. Normal and leukemic monocytes [illegible]

prominent phagocytic activity both in the cytologically mature and immature types *In vivo* phagocytized materials include erythrocytes and thrombocytes [27] *in vitro* uptake of latex particles occurred as well as uptake of cell debris in skin window exudates Transformation of HC into macrophages did not occur in skin window exudates in contrast to the early observable transformation of leukemic monocytes into large and highly phagocytic macrophages [55] Both normal and leukemic monocytes have been shown to exhibit subclass specific IgG receptors on their surface [25 56] but constant demonstration of these receptors in all HC failed in the HCl [23] In accordance with the absence of cellular LYS in the HC the urinary excretion of the enzyme was reduced or normal in patients with HC leukemia whereas high excretion of LYS seems to be typical for mature monocytic leukemia [4 56]

RS and RS cell leukemia Prior to discussion of the cytological relationship occurring among monocytic or HC leukemias and the RS cells the materials studied for the latter group should be briefly reviewed Diagnosis of RS was made from lymph node sections Imprints from these lymph nodes and from the pleural effusions of one patient showed closely resembling cells The cytochemically demonstrable hydrolytic enzymatic equipment of these cells was uniformly poor The very weak AcP and r N AS E activities were probably related to the ultrastructurally poorly developed GERL of these cells which also generally showed poor cytologic maturation The faint staining of small cytoplasmic vacuoles with SuB suggests the storage of lipids It is worth to mentioning that pyroninophilia was constantly very marked in the RS cells whereas PAS positivity was found to be variable although usually rather weak [64] Aspirated cells infiltrating the bone marrow in a case previously identified as RS of the bone consisted of a nearly identical cell population which differed only in the more intense activity of AcP and in the still more prominent pyroninophilia and PAS positivity Further approaches to the cytological characterization of these cells had not been made

Among 295 cases of acute leukemia we never had observed cases wich reasonably could be termed as RS cell leukemia In 4 patients primary localized tumors were originally identified as RS However the subsequently arising leukemias have been classified as lymphosarcoma cell leukemias as undifferentiated or as acute myeloblastic leukemias and because no reason existed to assume two independent malignancies we concluded that a primary tumorous manifestation of the leukemic process had occurred A further case originally diagnosed as reticulosis following the examination of a bone marrow biopsy clearly belonged to the myelogenous leukemias presenting both monocytic and promyelocytic features in the same leukemic cells

This case has been separately published together with three cytologically similar cases [55].

HC leukemia and RS. As far as the cytochemical pattern is concerned, differences among RS cells and HC are merely quantitative – for instance as the activities of AcP and α-N-E are concerned. Because of the weak activity of AcP the L+-tartrate resistance is difficult to evaluate in RS cells, however, distinct reduction of AcP activity occurred in most cases, whereas in HC the overwhelming activity of AcP is tartrate-resistant. Identical results have been reported also by KATAYAMA *et al.* [31].

The electron microscopic examination of the HC gives the impression of a rather differentiated cell. RC cells are distinctly less differentiated, do not show villiform cytoplasmic projections, and usually contain less mitochondria.

As previously reported, the immunocytological demonstration of IgG determinants in our hands revealed the presence – in decreasing order – of IgG, IgM and IgA on the surface of HC [8]. These data are in good accordance with the findings of other authors [10, 22] and suggest – although not conclusively – the B-cell nature of these cells [8, 10, 12, 22]. A further important finding which strongly supports this view has been reported by RUHL *et al.* [49], who were able to demonstrate IgG synthesis by *in vitro* cultured HC; however, these data have not been confirmed since that report. Recent studies suggest that at least a considerable part of the RS or histiocytic lymphomas also share typical B-cell features in that they are able to produce immunoglobulins [64] and that they bear immunoglobulin determinants and receptors of aggregated γ-globulin on their surface [2, 23, 24, 35]. Monoclonal gammapathias have been detected in 5 out of 140 patients who has RS and were studied by serum electrophoresis; peaks of IgG or IgM have been shown in 1 and in 4 patients, respectively [42]. A relationship of HC leukemia to the RES – as originally defined – cannot reasonably be affirmed. As we have outlined previously in this paper, the phagocytic ability of the HC is obviously poor and immaturity as the cause of poor phagocytosis is ruled out by the rather mature appearance of these cells under the electron microscope. In HC leukemia no involvement occurs of the cell types typically belonging to the RES. The cells in [illegible] [13] case are clearly different from HC.

Monocytic leukemia and RS or 'RS cell' leukemia. In introducing this paper we have already outlined that the theory of the reticuloendothelial origin of monocytes and monocytic leukemia continues to play a role in the nosological characterization of the monocytic leukemia or malignant reticulosis. Rela

tionship or cytological identity of 'histiocytic' or 'histioblastic' lymphomas or 'RS' to monocytic or monoblastic leukemia are discussed still in recent papers [6, 9, 37, 59]. Reticulin fibers as identifying features of tumors consisting of 'reticular' or 'reticuloendothelial' cell types seem to have little real value in that they can be observed in unequivocally myelogenous malignancies (see cases 3 and 6) as well as in HC-leukemia [14, 35].

Some authors have stressed the similarity of the cytochemical esterase pattern of RS, HC, and monocytes [37, 59]. However, as shown in table I, clear-cut qualitative differences can be shown among monocytes and the other cell types. Frequently, pitfalls in characterizing hemopoietic cells are due to the exclusive use of α-N-A as substrate for the demonstration of esterase activity; they can be overcome by using the N-AS E reaction, especially when in connection with the inhibition by NaF (table I). This staining procedure allows substantially better characterization of normal and leukemic monocytes, and misinterpretations due to insufficient techniques are avoided [54–56]. HUBER and FUDENBERG [25] reported on the presence of subclass specific IgG or Fc receptors on the surface of monocytes and macrophages. This technique has been successfully applied to characterize leukemic monocytes too [25, 56]. Some criticism has been made of the specificity of this method because presence of EA (i.e. IgG) receptors was also detected on B cells [38]. Receptors of the Fc fragment of aggregated Ig have been demonstrated on B cells as well [24]. However, by careful performance of these technique, blocking of the Fc receptors for EA complexes can be produced by preincubating the monocytes with IgG or subclasses of IgG, whereas no blocking occurred with the same concentrations of IgG in the demonstration of Fc receptors on B cells [23, 25], thus suggesting a qualitative difference among the receptors on these cells and monocytes. Following the conception of the 'mononuclear phagocytic system' (MPS) which recently has been put forward by VAN FURTH and THOMPSON [20], one could assume that 'RSs' could be derived from immature monocytes or monoblasts. However, the differences occurring among monocytes and RS cells argue also against this possibility.

Conclusions

Clear evidence against any cytological relationship among monocytic and HC-leukemia is given by cytochemical, ultrastructural, biochemical, immunologic, and functional criteria. In contrast to previous reports, which

have stressed the similarities of RS cells and leukemic monocytes, striking differences could be demonstrated at the cytochemical, ultrastructural, and immunologic levels which argue against a close relationship among these cells. Besides distinct ultrastructural differences, further gradual and some qualitative cytochemical differences were detected among leukemic HC and RS cells. However, remote relationship of HC and at least some of the RS cells can be assumed as far as the possible B-cell nature of these cells is concerned. The observed cases of 'RS cell leukemia' and 'reticulosis' actually had to be classified either as acute lymphatic or myelomonocytic leukemias. The terms 'reticulosarcoma' or 'reticulum' or 'reticulosarcoma cell leukemia' should be avoided and a more precise charcaterization should be afforded. The descriptive term 'hairy cell leukemia' should be preferred to the biologically inconsistent and historically erroneus term 'leukemic reticuloendotheliosis'. No actual biological relationship seems to exist among these malignancies and RES as it was originally described.

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Acta Haemat. 53: 277–284 (1975)

The Cytochemical β-Glucuronidase Reaction in the Differential Diagnosis of Acute Leukaemias[1]

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Key Words. Acute leukaemia · β-Glucuronidase · Cytochemistry · Lymphoblastic leukaemia

Abstract. The value of the cytochemical β-glucuronidase (β-gluc) reaction in the differential diagnosis of acute leukaemia was assessed in a series of 100 adult patients. A purely granular type of reaction was observed in 7 out of 8 cases of lymphoblastic leukaemia and in 2 of 11 cases of acute leukaemia of uncertain type. Such an exclusively granular reaction was never seen in other types of acute leukaemia. In most cases of myeloblastic, promyelocytic, myelomonocytic and monocytic leukaemia a positive staining reaction was noted which was either diffuse or a combination of diffuse and finely granular. The cells of one patient with lymphoblastic leukaemia were negative for β-gluc. A coarsely granular PAS reaction was n[illegible] e[illegible] [illegible] cases of lymphoblastic leukaemia including the one with negative β-glu[illegible] [illegible]n. Our results show that the β-gluc reaction is of definite value in the dia[illegible] [illegible] lymphoblastic leukaemia and that it is probably more sensitive than the PA[illegible] [illegible]n. In monocytic or myelomonocytic leukaemia, the pattern and inten[illegible] [illegible] did not differ appreciably from that seen in myeloblastic leukaemia.

The diffe[illegible] [illegible]s is still difficult in some cases of acute leukaemias. A p[illegible] [illegible] reaction for myeloperoxidase (perox), for naphthol AS [illegible] [illegible]ase or with the use of Sudan Black B (Sudan) is g[illegible] [illegible] as proof of the myeloid (granulocytic or monocytic) orig[illegible] [illegible] cells [11]. A strong nonspecific es[illegible] [illegible]e (est) react[illegible] [illegible] acetate or naphthol AS are used as substrate [illegible] characteristic for mature and imma[illegible] monocytes [5, 11] [illegible] considered to be negative for Su[illegible]

[1] Th[illegible] study was [illegible] [illegible] The Sigrid Jusélius Foundation [illegible]

dan and for the perox and chloroacetate esterase reactions, but because some myeloblasts are also negative for these reactions, the value of a negative reaction is equivocal Hence, a reliable positive staining method for lymphoblasts would be of decisive value In many laboratories, the periodic acid-Schiff (PAS) reaction is used for this purpose A coarse granular staining with the PAS reaction is considered characteristic for cells of lymphocytic origin whereas myeloblasts are either completely negative or weakly positive [3, 10], with staining in the latter case being faintly diffuse or discretely granular The PAS reaction is clearly a valuable tool in the differentiation of acute leukaemias but, because blast cells are negative in some cases of lymphoblastic leukaemia, and because the distinction between the small granules in myeloblasts and the large granules in lymphoblasts can be subtle, doubtful cases still remain Furthermore, some authors, e g, LEDER [5] and SCHMALZL and BRAUNSTEINER [11], have found large PAS positive granules in leukaemic cells not belonging to the lymphocytic series

In their extensive studies of β-glucuronidase (β-gluc) activity in leukocytes, LORBACHER [7] and LORBACHER *et al* [8, 9] found that most normal myeloblasts were negative Other cells of the granulocytic series were positive, with myelocytes staining more intensely than mature cells A strong reaction was also observed in monocytes In the granulocytic series, as well as in monocytes, the staining pattern was either diffuse, or diffuse and granular, but never exclusively granular An intense staining reaction was noted in the leukaemic cells of patients with myelomonocytic leukaemia [7] Most normal lymphocytes were found to contain β gluc -positive granules, distributed either as a few coarse clumps or as many small granules, but a diffuse reaction was never seen In most cases of chronic lymphocytic leukaemia, the percentage of negative lymphocytes was higher than in normal blood [9] In cases of acute lymphoblastic leukaemia LORBACHER [7] found a granular staining product in at least 10–20% of the blast cells

In the present investigation the β-gluc reaction was studied in blood and bone marrow smears from adult patients with acute leukaemia to obtain answers to the following questions Are β gluc -positive lymphoblasts always present in acute lymphoblastic leukaemia? Is the staining pattern of such lymphoblasts different from that of other immature leukaemic cells? Is the β-gluc reaction of value in the diagnosis of lymphoblastic leukaemia? Is the β gluc reaction useful in the diagnosis of monocytic and myelomonocytic leukaemia?

Patients and Methods

The series comprised 100 consecutive cases of adults suffering from acute leukaemia and treated at various hospitals belonging to the Finnish Leukaemia Group project [1]. Blood and bone marrow smears stained with May-Grünwald-Giemsa (MGG) were independently evaluated by three haematologists. In addition the following special stains were used in most cases: peroxidase by the method of Kaplow [4] with safranin omitted from the solution and MGG used as contrast stain; Sudan [?]; naphthol AS acetate esterase (est.) [?] and PAS as modified by McManus' method [12]. The β-glucuronidase (β-gluc.) reaction, as described by Lorbacher *et al.* [9], was studied in all 100 cases. Because this reaction was the subject of our study, it was not used as a diagnostic criterion.

The diagnoses were made as follows:

Acute myeloblastic leukaemia (AML) includes cases in which morphological changes were apparent in erythroblasts and megakaryocytes, definite myeloid pre-granulation present in some or all cells, or Sudan or peroxidase positivity.

Promyelocytic leukaemia (PML): promyelocytes, heavily granulated, were the predominant cell type.

Monocytic leukaemia (MoL): large numbers of immature monocytes or monocytoids in the blood or in the blood and bone marrow with at least 50% of the marrow immature cells heavily est. positive (4–5+ on a scale of 0–5).

Myelomonocytic leukaemia (MML): immature monocytes or monocytoids in the blood or marrow or in both with 20–50% of the immature cells heavily positive for est. In most of these cases, pathological changes were noted in erythroblasts or megakaryocytes, or in both.

Acute lymphoblastic leukaemia (ALL): unanimous opinion of the three haematologists; cells completely peroxidase and Sudan negative.

Acute leukaemia of undetermined type (LAL): cells negative for Sudan and peroxidase; [illegible] among the investigators or unanimous opinion that the type in these cases [illegible] be determined on morphological grounds.

In [illegible] bone marrow smears stained for β-gluc. activity, the positive staining [illegible] was bright red. As suggested by Lorbacher *et al.* [?], the staining pattern [illegible] as granular, diffuse or granular and diffuse. The strength of the [illegible] was arbitrarily graded on a scale of 1–5. Doubtful (±) reactions [illegible] as negative.

Results

Most [illegible] 72 out of 100 suffered from AML. In about three-fourths [illegible] blast cells showed a positive β-gluc. reaction (table I). Th[illegible] was usually weak, but some intensely staining blast cells (4–5 [illegible] in 13 cases. In all positive cases the predominant [illegible] diffuse, a bright [illegible] positive cases [illegible] ing and a granular staining were among

Table I The β-glucuronidase reaction in acute leukaemia

Diagnosis	Number of cases	β-Glucuronidase staining				
		negative	positive, diffuse	positive, diffuse and granular	positive, granular	strongly positive (4-5+)
AML	62	15	32	15	0	13
PML	4	1	1	2	0	1
Mon L	10	1	6	3	0	3
MML	5	0	3	2	0	2
ALL	8	1	0	0	7	6
UAL	11	6	0	3	2	1
All cases	100	24	42	25	9	26

Table II Comparison of the β-gluc and PAS reaction in ALL and UAL

	β-gluc		PAS	
ALL, 8 cases	granular	7	coarsely granular	4
			negative	3
UAL, 11 cases	negative	1	coarsely granular	1
	granular	2	negative	2
	diffuse and granular	3	negative	1
			not done	2
	negative	6	finely granular	4
			negative	2

ent (fig 1) An exclusively granular reaction was not encountered in any case of AML In 15 cases moreover the blast cells were completely negative

In 3 out of the 4 cases of PML the leukaemic cells were β-gluc -positive with staining either diffuse, or diffuse and granular.

The leukaemic cells were β-gluc -positive in almost all cases of Mon L and MML. They were completely negative in only one case of Mon L. The positive staining pattern, either diffuse, or diffuse and granular, was considered strong in 5 cases (3 of Mon L and 2 of MML) A purely granular reaction was not seen in these two groups

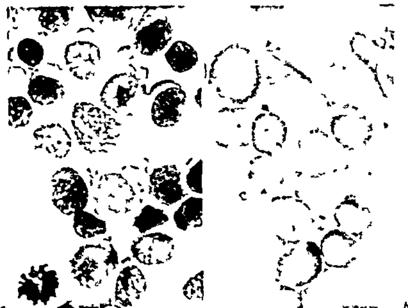


Fig. 1. AML, bone marrow. *a* MGG; *b* β-glu... ×...

The morphological diagnosis was ALL in 8 cases. The blast cells of 1 of these patients were completely β-gluc negative, whereas positive cells were noted in the other 7. The staining pattern was granular in all 7 cases and considered strong in 6 (fig. 2). Large red clumps or coarse granules predominated in 5 of the positive cases, and groups of small red granules in the remaining 2. No diffuse staining reaction was seen in this group.

The morphological type of acute leukaemia remained uncertain (UAL) in 11 cases. No β-gluc reaction was noted in 6 of them. A granularly positive reaction was recorded in 2 cases, and a combined diffuse and granular staining in 3.

A comparison of the results obtained with the β-gluc and the PAS reactions in ALL and UAL is reported in table II. A coarsely granular (lymphocytic) PAS staining was seen in 5 cases of ALL, one of them β-gluc negative, and in no case of UAL. The β-gluc positive cells were considerably more numerous than the PAS-positive cells in 4 cases of ALL.

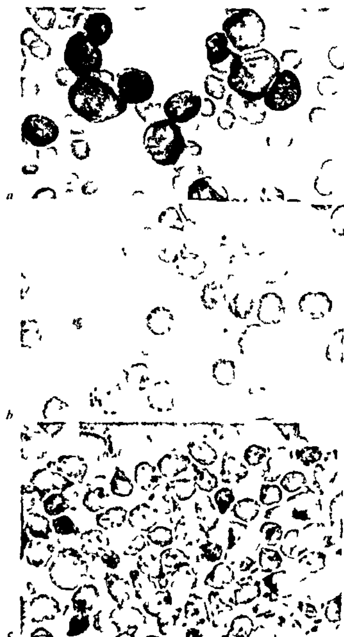


Fig 2 ALL *a* Blood MGG *b* Blood *β* gluc *c* Bone marrow *β* gluc (all pictures from the same case) × 700

Discussion

The evaluation of a cytochemical procedure for the identification of immature cells requires that a reliable diagnosis be made by other means. To be as certain as possible about the diagnoses in the present study we grouped together all doubtful cases as uncertain (UAL). Assuming that the diagnosis was correct in the remaining 89 cases, we can certainly in agreement with LORBACHER [7] and LORBACHER *et al.* [8, 9] conclude that an exclusively granular staining product in the β-gluc. reaction is specific for cells of the lymphocytic series. By this method 7 out of the 8 morphologically identified cases could be diagnosed as lymphoblastic leukaemia. Moreover, on the basis of the PAS reaction a correct diagnosis was possible in 5 of these cases. The β-gluc. reaction thus seems to be the more sensitive of the two reactions. However, because the blast cells were PAS-positive in the one β-gluc. negative case the use of both methods is recommended.

In the 11 cases of UAL, 2 had granularly β-gluc. positive blast cells, and we may now retrospectively assume that they were in fact lymphoblastic leukaemias. The PAS reaction was negative in both cases. The finely granular PAS staining product seen in 4 other cases in the UAL group was not specific for any cell type [3, 6, 11]. In 3 cases of UAL the blast cells showed a combined diffuse and granular β-gluc. reaction and hence they were probably cases of myeloblastic leukaemia.

The staining pattern of β-gluc. positive cells was similar in AML, MML and [illegible] either diffuse or a combination of diffuse and granular. A str[illegible] 4, 5 [illegible]) reaction of this type was seen in 3[illegible]% of the cases of M[illegible] [illegible]% of the cases of MML and in 22% of the cases of AML [illegible]rence in staining among these groups was not significant, howe[illegible] [illegible]refore the β-gluc. reaction could not be used in the different[illegible] [illegible] leukaemias.

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Acta haemat. 53: 285-291 (1975)

Functional Heterogeneity of the Transport Iron Compartment

I. *In vivo* Radoiron Clearance from High and Low Saturated Transferrin

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Key Words. Iron metabolism · Serum iron · Transferrin

Abstract. Two transferrin solutions of 4 and 80% ^{59}Fe saturation respectively were administered to humans 30 min apart. The radoiron clearance from the high saturated preparation was significantly accelerated. Comparable results were obtained in rats when normal rat serum was used instead of purified transferrin.

The two iron atoms bound by each transferrin molecule possess equal affinities to the two metal binding sites. This is indicated by equilibrium dialysis studies [1] and by the statistical distribution of iron when mixed with excess transferrin [2]. The extremely high affinity constant between the carrier and its metal at pH 7.5 excludes a dissociation controlled iron to cell transfer mechanism [6]. It is generally assumed that the transferrin molecule is taken up by specific membrane receptor sites, thereby undergoing conformational changes which facilitate the iron release; thereafter the carrier protein is shunted back into the surrounding medium [4, 12].

Current models of internal iron exchange are based on the existence of a homogenous plasma iron pool [7]. But recently, in *in vitro* experiments, FLETCHER and HUEHNS [10] produced evidence for functional differences between transferrin molecules carrying one or two iron atoms [9], a finding not confirmed *in vivo* so far [14]. Further testing of this phenomenon forms the basis of this communication.

Material and Methods

Preparation of iron transferrin solutions. 99% purified human transferrin (Behringwerke) was dissolved in saline in ratios of 0.5-3 mg/10 ml. The iron binding ca-

pacity was calculated to be 1.28 μg/mg protein on the basis of a molecular weight of 80000. All work was done under atmospheric conditions. $^{59}FeCl_3$ (SA 10–17 mCi/mg, Reaktor AG, Würenlingen, Switzerland) was obtained in 0.1 N HCl. The desired amount of radioiron was added stepwise to the transferrin solution in amounts of approximately 0.05 ml. The pH of the mixture was kept between 6.0 and 8.0 by small additions of 0.1 N HCl and 0.1 N NaOH. Each addition was followed by vigorous shaking; at the end the solution was exposed to open air for 30 min providing the bicarbonate necessary for stable iron binding by transferrin [3]. The total volume of acid and alkaline added did not exceed 3% per volume even when the transferrin was fully saturated with iron. If transferrin was loaded with $^{55}FeCl_3$ an identical procedure was followed. All solutions had a pH of 7.4 adjusted as above. In some experiments 10^{-2} M Na citrate dissolved in saline was added to the transferrin solutions to give a final citrate concentration of 10^{-3}–10^{-5} M. All solutions studied *in vitro* or administered to rats were provided with 200 U penicillin G Na/ml. In human experiments the preparations injected contained 200000 U of penicillin G Na.

Iron binding to transferrin. In order to check for undesired binding of radioiron to contaminants of the transferrin preparation the following experiments were carried out. (1) Electrophoresis on cellulose acetate, pH 7.4, demonstrated all radioactivity in the β_1 position of control plasma. (2) Since the transferrin preparation is contaminated with EDTA (0.003% per weight, thus giving a molar ratio of EDTA to transferrin of 1:127, communicated by Behringwerke), electrophoresis on thin-layer chromatography (Cellulose Merck) plates was performed. 0.01% solutions of transferrin in saline giving a pH of 7.4 were mixed with different amounts of radioiron and subsequently run at 500 V and 10 mA for 60 min at different pH values in a phosphate, pyridine and ammonium acetate buffer system, respectively. After staining the distribution of radioactivity was registered (Thin Layer Scanner II B, Berthold, model LB 2723; Autochron Recorder Berthold; rate meter integrater Berthold model LB 241K). ^{59}Fe-EDTA control solutions were run in parallel on the same plates. At 5 and 90% ^{59}Fe transferrin saturations and pH values of 7.4 and 6.4 all radioactivity was located within the transferrin band. If the saturation level was raised to 130% or the pH lowered to 4.75 some radioactivity migrated towards the cathode but none was concentrated at the EDTA site.

Studies in humans. Eleven volunteers were fully informed about the experimental purpose of the planned studies. In all cases they explicitly agreed to participate in the present investigation. Within 1 h they had two subsequent ^{59}Fe clearance studies. First, approximately 5 ml of a transferrin solution of 4% ^{59}Fe saturation (3 mg transferrin/10 ml saline) were injected, corresponding approximately to 1 μCi radioiron. Over the next 30 min at least eight 5-ml blood samples were drawn, immediately followed by the injection of an identical transferrin preparation but saturated to 80% with ^{55}Fe. Blood was again collected over the next 30 min. In five control studies $^{56}FeCl_3$ was added to the 4% radioiron saturated transferrin to raise the saturation level to 80%. The plasma radioiron clearance curves were established as described by FINCH *et al.* [7].

Studies in rats. Instead of purified transferrin, pooled serum of sideropenic rats (transferrin saturation below 10%) was employed. Iron deficiency was produced by

repeated blood removal through heart puncture. Equal amounts of $^{59}FeCl_3$ (approximately 5 μCi/dose) were added to different serum volumes to rise the iron saturation to 35 and 85%, respectively. Immediately prior to injection through a jugular vein catheter the volumes were equalized by addition of original unlabeled serum [11]. Over 60 min 10 0.02 ml blood samples were collected from the catheter and immediately pipetted into 2 ml saline, the cells were washed twice and the radioactivity of the supernatant was measured. The study was terminated by drawing blood from the heart for serum iron assay. All animal work was done under ether anesthesia. In four separate experiments using four serum pools a total of 19 and 23 rats injected with one of the two preparations were studied in parallel.

General methods. Haematocrits were determined by the micromethod. Serum irons were assayed according to Morgan and Carter [15]. Radioactivity was measured in a Nuclear Chicago Gamma Well Type Scintillation Counter, model 2433. Iron free glassware was used throughout. All chemicals were of reagent grade. Statistical comparisons between group data were carried out by Student's t test.

Results

Studies in humans. Each individual experienced an accelerated radioiron clearance from the high saturated transferrin as compared to the low saturated preparation. This difference was not explained by the slight drop in serum iron concentration. In control subjects the clearance rates become identical in both studies (table I).

Studies in rats. A significant difference in plasma radioiron clearance rate between the two groups investigated was observed ($p<0.05$). Thus in the low and high iron saturation studies the average $t_{1/2}$ were 83 and 65 min, respectively, while the mean serum iron levels and haematocrits were almost identical (table II).

Discussion

This study was designed to test in vivo the in vitro observation of a preferential incorporation of iron by reticulocytes from transferrin molecules carrying two iron atoms rather than one [8, 16]. First, a group of subjects had two subsequent ferrokinetic studies using a commercial transferrin preparation of 40% radioiron saturation for the first study and 80% saturation for the second. As iron attachment to transferrin binding sites occurs at random [2], in the former preparation molecules carrying one iron atom outnumbered fully loaded molecules, which on the other

Table 1 Individual subjects after having had two subsequent ferrokinetics within 1 h

No	Sex	Age	Clinical diagnosis	Haematocrit %	Study A[1] serum iron µg/100 ml	Study A[1] t ½ min	Study B serum iron µg/100 ml	Study B t ½[2] min
Experimental group								
1	M	68	Christian Weber's disease	35	43	49	37	38
2	M	52	aortic valvular disease	44	70	79	72	49
3	M	68	arteriosclerosis	42	100	135	95	48
4	M	54	diabetes mellitus	40	77	122	63	75
5	M	59	aortic valvular disease	42	75	98	58	69
6	M	57	peptic ulcer	39	87	66	77	46
Mean ± 1 SD					75±19	92±33	67±20	54±15
Control group								
1	M	50	mitral valvular disease	34	24	36	25	33
2	M	60	arteriosclerosis	41	30	33	27	33
3	F	71	arteriosclerosis	51	56	36	57	36
4	M	58	thrombosis	43	50	44	47	50
5	M	60	atrophic gastritis	46	73	62	69	62
Mean ± 1 SD					47±20	42±12	45±19	43±13

[1] The difference between study A and B consisted in differences of the radioiron transferrin saturation levels, being 4 and 80%, respectively. In control subjects (study A) the transferrin saturation was raised to 80% by the addition of cold iron.

[2] No correction was made for the radioactivity still present from study A.

hand, predominated in the latter. Indeed, in each individual investigated, radioiron clearance from the saturated transferrin preparation was accelerated unequivocally as compared to the low iron probe. Moreover, in a control group the clearance rates became equal after cold iron had been added to the low radioiron preparation. Second, groups of rats were injected with one of two homologous serum preparations identical in ^{59}Fe, ^{56}Fe and transferrin concentration containing either a small proportion of fully loaded ^{59}Fe transferrin in presence of a large excess of apotransfer-

Table II. Rats were injected with sideropenic rat serum brought to transferrin saturation levels of 15 and 85%, respectively; each experiment was carried out simultaneously in the high and low iron saturation recipients and was based on four different serum pools

Experiment	15% saturation			85% saturation		
	Haematocrit, %	serum iron µg/100 ml	t½ min	Haematocrit, %	serum iron µg/100 ml	t½ min
1	47	150	82	45	136	98
	46	146	95	47	160	81
	46	152	75	45	89	66
	47	118	95	46	134	124
				42	141	97
				45	149	104
2	42	190	132	47	192	65
	44	188	86	47	275	46
	44	214	136	47	310	48
	47	225	108	47	182	81
	47	251	116	43	217	31
	47	202	82	45	233	44
3	45	149	112	46	147	36
	47	137	87	44	159	50
	45	140	19	45	129	31
	46	143	55	46	121	42
	44	139	41	46	161	37
				45	132	49
4	52	192	47	50	176	45
	44	164	56	45	136	21
	45	193	18	45	142	40
	47	124	111	47	184	31
				47	229	143
Mean ± SD	45 ± 3	177 ± 40	81 ± 19*	46 ± 2	171 ± 52	65 ± 22*

* $p < 0.05$

tin, or predominantly the monoferric transferrin type. The plasma clearance experiments conducted with these preparations confirmed the data recorded in human subjects.

These findings are difficult to explain other than by an accelerated tissue uptake of iron bound to fully saturated transferrin molecules as compared to the half saturated species. Variable affinity to membrane re-

ceptor sites of the transferrin molecule according to its state of iron saturation could explain this finding [8, 13]. The association rate between reticulocytes and transferrin molecules complexed with one or two iron atoms has been found identical, however [5]. Therefore, the efficiency of the iron to cell transfer may be critically influenced by the degree of iron load of the carrier protein. Reflux of transferrin still carrying its original iron from reticulocytes has in fact been demonstrated [16].

The question may arise if impurities of the commercial protein preparation, EDTA in particular, have contributed to the results obtained. Taking into account a ratio of iron binding sites between EDTA and transferrin below 1:250, and the greatly differing affinity constants [2], this possibility appeared unlikely. Also, significant iron binding to EDTA was excluded by thin layer chromatography. Further, using a similar approach as described herein, FLETCHER [9] was unable to detect unequal clearance rates from 3 and 60% saturated transferrin, respectively. But, theoretically, at 60% saturation the proportion of fully saturated transferrin molecules was relatively small.

Acknowledgements. This investigation was supported by grants from the Schweizerischer Nationalfonds zur Förderung der wissenschaftlichen Forschung (3.89.69, 3.393.70 and 3.7710.72) and the Hartmann Müller-Stiftung der Universität Zürich.

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embryonic tissues and to elucidate the structural features of its accumulation at the cellular level.

Material and Methods

Animals

Female C_{57}Bl/6J inbred mice aged from 4 to 6 weeks, obtained from the Weizmann Institute of Science, Rehovot, Israel, were hormonally primed [5] and mated with 3- to 6-month-old males of the same strain. The morning after mating was designated as day 0 of gestation [1]. The gestation period of this strain of mice is 20–21 days.

Anemic mice: a second group of mice was bled repeatedly till the average level hemoglobin fell to 70% of that of the control group.

Both groups were given intraperitoneally 1.7 mg of ^{59}Fe-Imferon (82 mCi/ml, obtained from the Radiochemical Centre, Amersham, England) on day 10 of gestation. The animals were killed between the 12th and 15th gestational day. The bone marrow, livers, spleens and peripheral blood of the pregnant animals and the embryonic livers, spleens and peripheral blood were examined. Embryonic livers and spleens were removed under a dissecting stereomicroscope on sequential days of fetal development beginning on gestational day 12. Embryonic blood was collected in Petri dishes containing 1:1 Tyrode's solution and fetal bovine serum (Microbiological Associates, Bethesda, Md.) by heart puncture.

Electron Microscopy

Tissues for electron-microscopic examination were immediately transferred to cold 3 percent phosphate-buffered glutaraldehyde solution (pH 7.2), postfixed in 1 percent osmium tetroxide, dehydrated in ethanol and embedded in Epon 812. Thin (500–700 Å) sections were cut on an LKB Ultratome III, stained with uranyl acetate and lead citrate and examined with a Philips 300 electron microscope. Specimens for demonstration of Imferon structure were obtained by placing a drop of diluted Imferon (1:50 in distilled water) onto a formvar-coated grid.

^{59}Fe-Imferon Uptake in Pregnant Mice

Livers and spleens. Portions of livers and whole spleens were weighed and transferred to tubes containing 1 ml Tyrode's solution and homogenized. 0.25 ml of the homogenate was transferred into glass vials (Packard Instr. Co., Downers Grove, Ill.). 1.5 ml Soluene TM 350 (Packard Instr. Co.) and isopropanol (1:1) and 0.5 ml 30 percent perhydrol were added into the vials. They were incubated for 10 min at room temperature and for an additional 10–15 min at 40 °C. After addition of the scintillation mixture (1:8 0.5 N HCl and Instagel (Packard Instr. Co.)) the vials were kept overnight in the dark at 4 °C. The activity of the dissolved tissues was counted using a liquid scintillation spectrometer (Packard Instr. Co., model 3375). The results are expressed in counts per minute for each organ of a single animal. Comparable experiments were carried out using identical amounts of tissue.

Bone marrow. Femoral bone marrow pooled from 5 animals was resuspended in 1 ml 1:1 Tyrode's solution and fetal bovine serum. The cells were counted, the vol-

Acta haemat 53 292-299 (1975)

Transfer of Iron-Dextran Across the Placenta

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Key Words Anemia Imferon® Iron in pregnancy Iron-dextran Placental passage of iron

Abstract In pregnant mice ^{59}Fe labeled iron-dextran (Imferon®) is transferred across the placenta It was detected in the bone marrow, liver spleen and peripheral blood of the pregnant animal as well as in the embryonic liver erythroid precursors and peripheral blood Uptake by liver and peripheral blood cells of pregnant anemic mice and by liver erythroid precursors of anemic embryos was significantly higher than in normal control animals Electron microscopic examination revealed that the iron deposits in the embryonic liver erythroid precursors had the same structure as the injected Imferon

Iron dextran (Imferon®, Fisons Ltd, England) is widely acknowledged to be an effective drug in the treatment of iron-deficiency anemia [6] While the beneficial results of the drug in anemia of pregnancy are frequently reported, studies on its placental transfer are scarce COX *et al* [3] studied the serum iron level and iron-binding capacity in progeny of mothers given iron-dextran infusions and demonstrated that there is no passage of iron-dextran across the placenta once the maternal serum iron level has returned to normal When the serum iron level is at its peak after infusion of the total dose, a small amount of iron is believed to cross the placenta possibly as unchanged iron-dextran, but the iron stores of the progeny do not contain excessive amounts of iron On the other hand, COTES *et al* [2] showed that 0 25–4 5% of ^{59}Fe labelled iron-dextran administered to pregnant monkeys was recovered in the fetuses

The aim of the present study is to examine the transfer of Imferon through the placenta to follow its mode of deposition in maternal and

Table I. Uptake of ^{59}Fe-Imferon by tissues of pregnant anemic mice on different days of gestation

Tissues	1?th day		13.5 day		14.5 day		15th day	
	A	C	A	C	A	C	A	C
Liver: cpm/g	[illegible]	53,619	130,234	[illegible]	[illegible]	92,940	117,670	[illegible]
Spleen: cpm/g	[illegible]	31,473	61,432	51,571	[illegible]	[illegible]	50,258	[illegible]
Bone marrow cpm/1.7 × 10 cells	[illegible]	[illegible]	[illegible]	[illegible]	223	[illegible]	225	205
Peripheral blood cpm/0.1 ml packed cells	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

A = Anemic mice, C = control mice

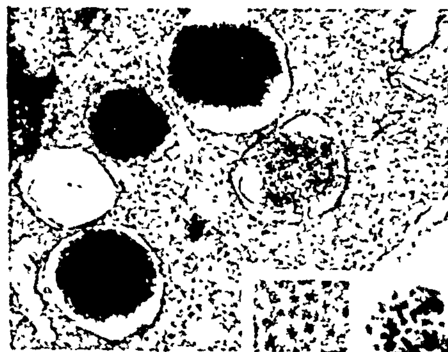


Fig. 1. [illegible]

pension centrifuged and after discarding the supernatant the cells were again suspended in 0.25 ml of the same mixture and transferred into vials for counting.

Peripheral blood. Heparinized blood was obtained by heart puncture. The cells were washed three times with 0.9 percent saline solution. Packed cells (0.1 ml) were transferred into vials and their activities counted as previously described.

⁵⁹Fe Imferon Uptake in Embryos

Livers. Whole embryonic livers extracted on gestational days 12–15 were cut into small pieces, placed in 1:1 Tyrode's solution and fetal bovine serum and disaggregated by squeezing through a fine tipped Pasteur pipette. Activity of the cells was then counted as previously described.

Peripheral blood. Peripheral blood from embryos of gestational days 12–15 was pooled, the cells counted, sedimented and 0.1 ml of packed cells transferred into vials for radioactive counting.

Results

Mothers. Uptake of ⁵⁹Fe-Imferon in tissues of anemic mice is recorded in table I. In both the anemic and control animals, activities are significantly increased on day 13 of gestation, i.e. 3 days after injection of Imferon, as compared with uptake on day 12 ($p<0.005$). In the anemic mice there was a twofold increase in uptake by the spleen between days 12 and 13; in the control animals, spleen uptake increased with gestation time. While uptake in the bone marrow of both anemic and control mice was more or less steady, the peripheral blood cells showed a marked increase in the uptake during gestation, which was much more pronounced in the anemic animals ($p<0.005$).

Embryos. As the gestation period progressed, in both anemic and control embryos there was a marked increase in the Imferon uptake by the liver, which was even more pronounced in the anemic fetuses ($p<0.01$); uptake by the peripheral blood was approximately constant.

Electron microscopy. Deposits of iron were easily detected in the liver red cell precursors of both anemic and control mice, but in much greater amounts in the embryos of anemic mothers. They were found in precursors of different gestational stages, but appeared to be most abundant in liver red cell precursors of the 13th day. The iron deposits were visible mainly in the poly- and orthochromatic erythroblasts, located in large clusters surrounded by membrane (fig. 1). Their structure suggested the presence of ferritin granules, but we were not able to show the typical configuration of the ferritin molecule. On the other hand, the structures appeared compatible with those of the Imferon molecule (fig. 1, insert).

Table 1. Uptake of [59]Fe Imferon by tissues of pregnant anemic mice on different days of gestation

Tissues	12th day		13th day		14th day		15th day	
	A	C	A	C	A	C	A	C
Livers, cpm/g	77,4[illegible]	33,619	130,234	100,236	121,5[illegible]	92,940	117,670	110,927
Spleens, cpm/g	[illegible]	38,423	63,432	51,571	[illegible]	57,731	50,234	61,056
Bone marrow cpm/3.7 × 10 cells	[illegible]	168	317	131	221	1[illegible]6	225	205
Peripheral blood cpm/0.1 ml packed cc	1[illegible]	1,116	4,70[illegible]	2,[illegible]0	6,82[illegible]	3,0[illegible]	6,070	[illegible],313

A = Anemic mice [illegible] mice



Fig. 1. [illegible] ... the liver of a 13 day ane[illegible] ... embryo ... deposits ... membrane ... liver ... as compared with the ... of Imfer[illegible]



Fig 2 Iron deposit in the nucleus of a polychromatophilic erythroblast × 73,000

Fig 3 Unstained thin section of a 13-day embryonic liver showing large amounts of iron deposits in an orthochromatic erythroblast × 20,000

Table II. Uptake of ^{59}Fe-Imferon by tissues of embryos from anemic mice at different gestational ages

Tissues	12th day		13th day		14th day		15th day	
	A	C	A	C	A	C	A	C
Livers, cpm/3.8 × 10^7 cells	916	578	1,122	509	1,211	893	1,311	896
Peripheral blood cpm/2.3 × 10^7 cells	–	1,536	2,132	1,875	2,310	2,042	1,456	2,807

A = Embryos from anemic mothers; C = control embryos.

On rare occasions, iron deposits were found even in the nucleus (fig. 2). The iron deposits were promptly detected in unstained thin sections, due to their electron density (fig. 3). They were not found in the parenchymal liver cells, nor in the mesenchymal cells of the embryonic spleens.

Discussion

The detection of radioactivity in the erythroid precursors of embryonic livers and peripheral blood confirms the transplacental transfer of the drug. The increased incorporation of radioactive Imferon in embryonic livers with advancing gestation may be explained by the increase in more mature erythroid precursors during embryonic development. While in the embryonic livers of the 11th day 50% of the erythroid precursors are proerythroblasts, by day 14 the proportion of these cells decreases to 5%; during the same period the proportion of more mature erythroblasts such as poly- and orthochromatic erythroblasts capable of synthesizing hemoglobin increases rapidly [7]. It was shown that the late erythroblasts and particularly the polychromatophilic erythroblasts are the most active in hemoglobin synthesis [8, 10] and in iron incorporation [8, 9].

The more pronounced uptake of radioactive Imferon in the livers of embryos of anemic mothers is rather surprising, since the anemia was mild and it is supposed that the iron content in the embryo was not reduced.

The steady uptake of ^{59}Fe-Imferon by the peripheral embryonic blood during the progress of gestation may be explained by the presence of ma

ture yolk sac cells in the peripheral blood which are not able to synthesize new hemoglobin

The electron-microscopic findings suggest that the iron deposits in the erythroid precursors of the embryonic livers are most probably Imferon. The similar structure of the Imferon molecule *in vivo* and *in vitro* (fig. 1) would support this indication. This observation is compatible with the findings of MUIR reported by COX [3] who demonstrated that infused human and rhesus throphoblasts contain particles similar in size and structure to pure iron-dextran which do not have the ultrastructure of ferritin or hemosiderin. However, the author could not find such particles in specimens obtained after iron dextran infusion.

The uptake of Imferon in the maternal tissues is as expected (except for the peak of radioactivity) found in the livers of both control and anemic animals at day 13 of gestation, i.e. 3 days after Imferon administration. *It is possible that by this stage of gestation, the liver, which is one of* the important organs for iron storage, is already iron-saturated and cannot store more.

Acknowledgment. Our thanks are given to Mrs. SONIA SELIGSON-ZINGER and Mrs. RIVKA REZNICK from the Department of Biostatistics, KUPAT HOLIM for their help in preparing the statistical data. This study was supported by a grant from FISONS Ltd. England and partially by a grant obtained from the Chief Scientist Bureau, Ministry of Health of which M. D. is an established investigator.

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Acta haemat 53 300-308 (1975)

In vitro and *in vivo* Influence of Penicillin G on Platelet Aggregation[1]

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Key Words Platelet aggregation · Platelet electrophoretic mobility · Platelet function · Penicillin G · Screen filtration pressure test

Abstract The influence of penicillin G on the *in vitro* and *in vivo* ADP induced platelet aggregation and on the platelet electrophoretic mobility has been studied. The *in vitro* investigations in presence of penicillin concentrations varying between *1 000* and *10 000* IU/ml of plasma and doses of adenosine diphosphate (ADP) between 0.3 and 25 μM/ml have shown that the photometric aggregation as well as the screen filtration pressure are both decreased according to the doses of antibiotic and ADP. The electrophoretic mobility is also impaired. The *in vivo* study of 8 patients submitted to massive penicillin therapy for bacterial endocarditis seems to be in agreement with the *in vitro* results and has shown a prolonged bleeding time, an hypoadhesiveness, an impaired photometric aggregation with an early disaggregation and a decrease of the screen filtration pressure and platelet electrophoretic mobility.

At the present time, many drugs are known to affect blood platelet functions, and, particularly their aggregability. However, very few reports have dealt with the possible interference of antibiotics with haemostasis [4, 6, 8].

Our study reports *in vitro* and *in vivo* investigations about the influence of penicillin G on some platelet functions. Such a work has been motivated by the observation of a patient who, being submitted to massive penicillin therapy for bacterial endocarditis, had haemorrhage dur

[1] This work was supported by Grant No. 74 34 202 00 450 75 01 of the DRME (Biological Section).

[2] We thank Mrs J. Dexer for her technical assistance.

ing dental extraction, his bleeding time was found to be very prolonged and platelet aggregation test altered. Then, other observations permitted us to find out the same changes associated with the same treatment.

Materials and Methods

Blood was drawn by clean venepuncture into plastic tubes containing sodium citrate 0.13 M (1 vol of anticoagulant and 9 vol of blood) and gently mixed.

For the *in vitro* studies, blood was collected from several healthy blood donors who had not ingested aspirin or any other drug for at least 10 days before testing.

In vivo studies were carried out on patients who presented subacute bacterial endocarditis and therefore intravenously perfused with highly concentrated penicillin G (1 000 [illegible] international units (IU)/kg body weight daily). One of them suffered from a [illegible] renal failure and for this reason, he received only 6 × 10⁶ IU/day, but his serum [illegible] concentrations were in the same range as the other patients (i.e. 50-2[illegible]).

All these pa[illegible] studied at least 3 days after the beginning of the treatment. In none o[illegible] we find any reason for a prolonged bleeding time. They did not re[illegible] drug known to interfere with platelet functions.

Controls con[illegible] platelet rich plasma (PRP) from healthy subjects studied in the same con[illegible]

For the *in* [illegible] studies, the PRP was prepared by slow centrifugation at 150 g at [illegible] temperature for 8 min, and diluted in the autologous platelet poor plasma [illegible] to obtain the same platelet concentration.

Coagulation [illegible] performed with the following tests [2]: bleeding time (according to I[illegible]), count using an electronic Coulter [illegible] Counter, whole blood clotting [illegible] (Wh[illegible]) prothrombin time (Quick), thrombin clotting time (Biggs an[illegible]) activated partial thromboplastin time (Larrieu and Weiland), leve[illegible] thrombometric method of Von Clauss), [illegible] the [illegible] prothrombin consumption after 4 h incubation at 37 °C.

Platelet ad[illegible] retention to glass beads was evaluated on whole venous blood [illegible] technique (normal range in our laboratory 25 ± [illegible]).

Platelet agg[illegible] using two techniques:

(a) the pla[illegible] has been carried out at 37 °C in an [illegible] aggregometer [illegible] of light transm[illegible] of PRP samples and continuous recording [illegible] aggregating agents were adenosine diphosphate (ADP) and co[illegible] measured were the deflection of the aggregation curve [illegible] at 45 sec, the rate of maximal aggregation and the e[illegible] expressed as the percentage of disaggregation [illegible] the PRP.

(b) the screen [illegible] test (SFP) has been performed using an apparatus described [illegible] recording [12]. This method consists in measuring the p[illegible] PRP forced at a steady rate through mesh

lic screens formed by multiple 20-μm^2 micropores This technique was applied to the PRP to which addition of ADP induces an increase in the SFP [12] due to the formation of platelet aggregates The results were expressed in mm Hg The measurements were made at different times after the addition of ADP The condition of experiments and the proportions were the same as those used with the photometric study

The platelet electrophoretic mobility (EM) was determined in liquid phase in an apparatus with a rectangular chamber [11] All the measures were performed in autologous plasma

Reagents ADP (Sigma Laboratories) used at final plasmatic concentrations of 0 3 to 25 μM/ml, collagen (Stago Laboratories) diluted in Tris buffer (ionic strength 0 15 M pH 7 4) and used at 50 μg/ml as a final concentration penicillin G For the *in vitro* studies sodium salt of benzylpenicillinate was used It was dissolved in distilled water, then diluted with Michaelis buffer (ionic strength 0 15 M pH 7 35) and finally PRP samples were incubated with various dilutions of penicillin (1 vol of antibiotic and 9 vol of PRP) at 37 °C for 10 min before the addition of ADP Final plasmatic concentrations of penicillin were 1 000 2 000 5 000 10 000 IU/ml Control samples consisted in the same PRP incubated with buffer in the same proportion, instead of penicillin

The patients were treated either with sodium or potassium salt of benzylpenicillinate

Results

In vitro *studies* The influence of penicillin on platelet aggregation with ADP and electrophoretic mobility is illustrated in figure 1 and table I As is obvious from table I, the relative EM of platelet suspensions in contact with penicillin is progressively decreased, according to the dose of antibiotic On the other hand, the SFP is significantly decreased This decrease becomes much more perceptible as the concentrations of penicillin arise We may notice also, that, for the lowest dose of antibiotic (2,000 IU) there is still a difference in the results of SFP, as regards the concentrations of ADP added, but, for high doses of penicillin (10,000 IU), the SFP becomes nearly equal to that of PRP alone, i e nearly without any aggregates in the suspension

Furthermore, a closely parallel inhibitory effect of penicillin on platelet behaviour is found out by the photometric study of ADP induced aggregation Figure 1 presents an example of aggregation curves recorded with the same PRP incubated with different concentrations of penicillin in the presence of ADP at 1 25 μM/ml, and points out the inhibitory effect in relation to the dose of this antibiotic In fact, this aggregation is all the more disturbed as the penicillin concentration is higher and the



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Table I In vitro influence of penicillin G on EM and SFP of platelets (SFP of PRP only 7 ± 3 mmHg)

Final concentrations of Penicillin G IU ml in PRP	EM, means of 8 measures	SFP, mmHg in presence of ADP, means of 4 measures					
		ADP, 0 625 μM ml			ADP, 0 875 μM ml		
		1 min	3 min	5 min	1 min	3 min	5 min
2,000	0 96 ± 0 05	18	14	12	36	25	21
5 000	0 91 ± 0 06	8	7	9	7	12	10
10 000	0 86 ± 0 06	7	12	10	12	7	6
0 (buffer instead of penicillin G)	1 01 ± 0 04	44	25	18	50	44	27

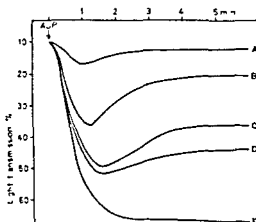


Fig 1 Example of ADP induced platelet aggregation in a normal PRP The PRP was incubated for 10 min at 37 °C before addition of ADP (1.25 μM ml) with penicillin, 10 000 IU ml (curve A) penicillin 5,000 IU ml (curve B) penicillin 2,000 IU ml (curve C) penicillin, 1 000 IU ml (curve D) and Michaelis buffer (curve E)

inductive dose lower Thus, for 10,000 IU, aggregation remains irreversible only for very important inductive doses (superior to 5 μM/ml) whereas controls disaggregate only for doses always below or equal to 0 625 μM/ml We observed then that the dose of 5,000 IU leads to a lesser inhibitory effect (and disaggregation appears at lower doses of

ADP), whereas concentrations of 2,000 and 1,000 IU/ml are less effective

As for the collagen-induced aggregation, we found that low concentrations had no significant effect On the contrary, in presence of 5,000 and 10,000 IU either no aggregation occurred, even after 6 min recording or, when it occurred, the latence time was very prolonged and velocity very low But, the rate of complete aggregation was finally normal

In vivo *studies* Our study concerning patients submitted to massive penicillin G therapy corroborates the *in vitro* results Bleeding time was prolonged in all of these patients (about 15 min and in 3 of them, it was longer than 20 min) with normal platelet count Platelet retention to glass beads was very low (2–12%) In the same way, ADP-induced platelet aggregation was markedly disturbed (table II) Disaggregation occurred very early, even for high inductive doses, it generally appeared already at 5 µM and irreversible aggregation was enhanced only by important doses (12 5–25 µM/ml)

Results from the *in vivo* collagen induced aggregation were less constant We found a slight decrease in the aggregation rate (15–40% diminution) In 4 of the patients, PRP velocity was significantly decreased (6–14%, control range 25–35%) When platelet function tests were repeated during the course of penicillin treatment, nearly the same results were obtained

On the other hand, we did not find any alteration of the platelet function tests in a patient perfused with usual concentrations of penicillin G (10×10^6 IU daily)

Intensive penicillin therapy did not affect the plasmatic coagulation tests which always remained within a normal range Prothrombin consumption time was not significantly altered

Platelet factor 3 availability was found normal in the 3 patients' plasmas studied

Discussion

The present study shows that high concentrations of penicillin G prolong the bleeding time and impair platelet adhesiveness to glass and platelet aggregation Our *in vitro* findings are in agreement with those of CAZENAVE *et al* [3] who reported that penicillin G, when used at 1,000–8 000 IU/ml inhibited the ADP-, collagen- and thrombin induced platelet aggregation and the release reaction Similar observations were

Table II ADP induced platelet aggregation and EM in patients submitted to penicillin therapy

Cases	ADP induced aggregation, ADP in μM ml							Electrophoretic Mobility
	25	12.5	5	2.5	1.25	0.625	0.312	
Patient 1, 250 000 P/mm³								
velocity, %	40	35	40	31 (57)	28 (55)	(53)	(40)	0.79 (1.00)
aggregation max., %	62	58	50	39 (71)	36 (70)	(64)	(48)	
disaggregation, %		46	52	78	78			
Patient 2, 250 000 P/mm³								
velocity, %		28 (35)		6 (35)		(21)		
aggregation max., %		42 (84)		16 (76)		(40)		
disaggregation, %		77		92		(86)		
Patient 3, 300 000 P/mm³								
velocity, %		28	24	24 (60)	17 (54)	13 (51)	(35)	
aggregation max., %		66	55	53 (77)	39 (71)	27 (52)	(43)	0.89 (1.01)
disaggregation, %					75	77	(84)	
Patient 4 200 000 P/mm³								
velocity, %	35	27	23 (44)	21 (44)	(44)	(29)	(7)	0.96 (10 days
aggregation max., %	51	55	44 (80)	35 (78)	(73)	(60)	(37)	after end of
disaggregation %		55	63	95			(65)	treatment)
Patient 5, 300 000 P/mm³								
velocity %	20	22 (53)	16 (46)	15 (40)	(36)	(25)		
aggregation max., %	51	49 (80)	42 (75)	32 (75)	(56)	(40)		0.87 (1.03)
disaggregation %			75	79		(85)		
Patient 6 300 000 P mm³								
velocity, %	33	33 (48)	31	31 (47)	25 (38)	(30)	(19)	
aggregation max., %	59	51 (84)	50	42 (77)	34 (74)	(57)	(36)	0.93 (0.99)
disaggregation, %	58	58	64	82	87		(77)	
Patient 7, 300 000 P mm³								
velocity, %	35	30 (58)	28 (59)	24 (50)	24 (48)	(43)	(33)	
aggregation max., %	55	55 (85)	53 (90)	39 (89)	27 (86)	(64–84)	(40)	0.82 (1.02)
disaggregation %	65	67	68	80	94		(70)	
Patient 8, 350 000 P mm³								
velocity %		55 (68)	50 (68)	40 (66)	29 (65)	(47)		
aggregation max., %		63 (80)	63 (83)	55 (73)	38 (70)	(44)		0.95 (0.99)
disaggregation, %			50	63	75	(91)		

Figures in parentheses are normal PRP values measured on the same day P = Platelet.

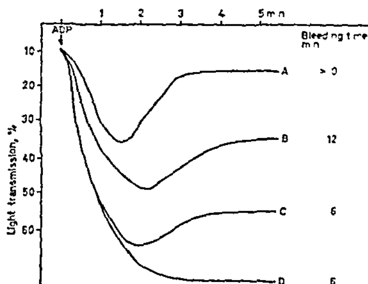


Fig 2 Bleeding times and photometric ADP (1.25 μM/ml) induced platelet aggregation curves in a patient's PRP at different times during penicillin treatment (curve A), 3 days after treatment (curve B), 6 days after treatment (curve C), before treatment (curve D)

reported *in vivo* by MC CLURE *et al* [9] with carbenicillin, (disodium salt of α-carboxypenicillin) when used at a high dosage

On the other hand, in contrast with the *in vitro* studies of FLEMING [5], we could not find any *in vivo* plasmatic coagulation change However, it is possible that the purity of the antibiotic may be an important factor to explain this discrepancy

The platelet function changes seem to occur very quickly after the onset of treatment as we found alterations as soon as the 3rd day Then, the modifications were present during all the treatment period with a progressive recovery of normal aggregation after the end of the treatment (fig 2) In all cases, 10 or 15 days after penicillin therapy had been stopped, bleeding time and platelet aggregation were strictly normal (whereas platelet adhesiveness was still slightly impaired) This fact might correspond to a complete renewal of the circulating platelet pool from the bone marrow

From our *in vitro* and *in vivo* studies, it appears that penicillin may act only at high concentrations, as usual treatment with 10×10^6 IU daily, did not influence platelet function

Our *in vitro* results, obtained after an incubation of only 10 min, confirm a direct inhibitory action of penicillin. However, there is a discrepancy between the *in vivo* and *in vitro* effects, according to the dose. As concerns clinical use, seric concentrations achieved were below 300 IU/ml, on the other hand, the *in vitro* ADP-induced aggregation in presence of 1,000 IU was less impaired than the *in vivo* aggregation for the same inducive dose. There might be several hypothesis for this fact: first, it may be suggested that, although penicillin is not very much degraded *in vivo*, some derivatives might have a stronger action than the antibiotic itself, requiring lower doses; second, under continuous perfusions, patients' platelets are suffusing in penicillin for long periods of time, and it may be assumed that this long contact time permits some interferences between certain particular structures of platelets and the antibiotic. The possible interferences with some membrane phospholipids might also be considered [10].

Besides this, the decrease of EM *in vitro*, after a short incubation time, emphasizes the fact that penicillin could have a direct effect on platelet membrane. The decrease observed might suggest that this antibiotic has an action upon some biochemical sites on the platelet surface (ionized groups or formation of an eventual positive complex between penicillin, plasma proteins and some membrane components). Although the mechanism by which penicillin interacts with platelet functions is not known at the present time, some suggestions can be made on the basis of this experiment and of observations with other cells [7].

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An *in vitro* Method to Study the Participation of Various Components in Autoimmune Hemolytic Anemia

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Key Words Autoimmune hemolytic anemias Osmotic fragility test Poliovirus III

Abstract A method is described which allows for the *in vitro* evaluation of various etiological components in autoimmune hemolytic anemia As an example of the applicability of the technique erythrocytes and serum from a patient with an autoimmune hemolytic anemia suspected to be due to poliovirus III were tested with poliovirus III and complement Incubation of the patient's cells with complement, poliovirus and autologous serum was required for optimal *in vitro* fragility The method is proposed as a practical and convenient *in vitro* technique for evaluating the participation of individual components in the pathogenesis of autoimmune hemolytic anemias

Cytomegalovirus Epstein Barr, influenza A, influenza C, herpes simplex and zoster hepatitis B coxsackie A and measles viruses have been implicated as etiological agents in autoimmune hemolytic anemia but the precise role of viruses in the erythrolysis has not been clearly established [7] The implication of various etiological agents in autoimmune hemolytic anemia has been limited by the lack of an *in vitro* correlate of *in vivo* hemolysis To this end we have adapted the erythrocyte fragility test, which has been shown to be a reliable *in vitro* model of *in vivo* erythrolysis [1] to investigate the possible participation of a viral agent and other components of erythrolysis in the pathogenesis of an autoimmune hemolytic anemia

The recovery of poliovirus III on two separate occasions during two hospitalizations from the stools of an unvaccinated 9 month-old male with a recurrent and fatal nonspherocytic 'warm type' autoimmune hemolytic anemia suggested the possible etiological participation of the vi-

rus in the hemolytic process. The patient's hemoglobin was consistently less than 10.0 g/100 ml with persistent reticulocytosis and the direct antiglobulin test was positive. However, there was no rise in serum antibodies to poliovirus type I, II, or III, the virus could not be recovered from the blood or bone marrow, and the IgG autoantibody did not demonstrate anti-poliovirus specificity. No other etiological agents or immunodeficiency state were demonstrable.

Since poliovirus has not been implicated in the pathogenesis of autoimmune hemolytic anemia, this case seemed suitable for the *in vitro* evaluation of the possible independent and collective roles of the virus, serum constituents, and erythrocytes in the production of erythrolysis. The demonstration of virus-mediated red cell instability *in vitro* may suggest a possible viral etiology in the patient's hemolytic illness.

Materials and Methods

Prior to therapy the patient's blood was collected by venesection under sterile conditions and defibrinated with glass beads. The serum was separated by centrifugation and frozen in sterile containers at −20 °C. Blood from a clinically healthy, compatible 12 month-old control subject was collected and fractionated similarly. The red blood cells (RBC) from the patient and control, collected 1 h prior to the incubation procedure, were washed three times with normal saline. Fox strain poliovirus type III ($10^{7.3}$ $TCID_{50}$/0.1 ml LLCMK2 cells) was obtained from the American Type Culture Collection. The virus preparation was found to have an osmolality of 280 mOs/l and a sodium concentration of approximately 150 mEq/l. Incubation mixtures consisting of 0.2 ml of patient or control serum, 0.2 ml of patient or control RBC, 0.2 ml of guinea pig complement (Hyland), and 0.05 ml of virus were prepared in duplicate. Final volume was made up to 0.7 ml with 0.9 percent saline. The mixtures were incubated for 20 h at 37 °C.

The osmotic fragility of the incubated cells was measured with the Erythrocyte Fragility Kit (Becton Dickinson). After thorough shaking, 20-μl aliquots of the incubation mixtures were delivered into a series of 10 reservoirs of ascending sodium chloride concentration (0.00%, 0.30%, 0.35%, 0.40%, 0.45%, 0.50%, 0.55%, 0.60%, 0.65%, 0.85%). After incubation at room temperature for 20 min, the contents of the reservoirs were centrifuged at 365 g for 5 min. The absorbance (A) of the supernatant fluid in each of the 10 cuvettes was measured at 540 nm in a Coleman Jr. spectrophotometer. The percentage of hemolysis was determined by the following formula

$$\text{Percent hemolysis} = \frac{A_x - A_{0.85\%}}{A_{0.0\%} - A_{0.85\%}},$$

where A_x is the absorbance of the tube with solution of sodium chloride

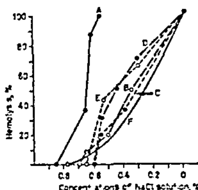


Fig. 1. Results of osmotic fragility tests performed on incubation mixtures prepared as indicated and incubated at 37°C for 20 h. Each point represents the mean value of duplicate determinations. Plus (+) indicates presence of the component, zero (0) indicates absence of the component in the mixture. See *Material and Methods* for concentrations of components.

Curve	RBC		Serum		Poliovirus	Complement
	patient	control	patient	control		
A	+	0	+	0	+	+
B	0	+	+	0	+	+
C	+	0	+	0	0	+
D	+	0	+	0	+	0
E	+	0	0	+	+	+
F	0	+	0	+	+	+

Results

As shown in figure 1, when the patient's RBC and autologous serum were incubated with complement and poliovirus III, there was a marked shift to the left of the osmotic fragility curve (curve A). When homologous, compatible erythrocytes were substituted for autologous RBC in the test system (curves B and F), there was no significant shift in fragility from the control cells regardless of the serum used. As can be seen from curves C and D, virus and complement were required for maximum fragility. When homologous serum was substituted in the test system (curve E), the osmotic fragility curve was shifted to the left, but not to the extent seen with autologous serum.

rus in the hemolytic process. The patient's hemoglobin was consistently less than 10.0 g/100 ml with persistent reticulocytosis and the direct antiglobulin test was positive. However, there was no rise in serum antibodies to poliovirus type I, II, or III, the virus could not be recovered from the blood or bone marrow, and the IgG autoantibody did not demonstrate anti-poliovirus specificity. No other etiological agents or immunodeficiency state were demonstrable.

Since poliovirus has not been implicated in the pathogenesis of autoimmune hemolytic anemia, this case seemed suitable for the *in vitro* evaluation of the possible independent and collective roles of the virus, serum constituents, and erythrocytes in the production of erythrolysis. The demonstration of virus-mediated red cell instability *in vitro* may suggest a possible viral etiology in the patient's hemolytic illness.

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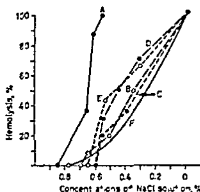


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E	+	0	0	+	+	+
F	0	+	0	+	+	+

Results

As shown in figure 1, when the patient's RBC and autologous serum were incubated with complement and poliovirus III, there was a marked shift to the left of the osmotic fragility curve (curve A). When homologous, compatible erythrocytes were substituted for autologous RBC in the test system (curves B and F) there was no significant shift in fragility from the control cells regardless of the serum used. As can be seen from curves C and D virus and complement were required for maximum fragility. When homologous serum was substituted in the test system (curve E), the osmotic fragility curve was shifted to the left, but not to the extent seen with autologous serum.

Discussion

The occurrence of a persistent hemolytic state in a patient from whose stools poliovirus III was repeatedly recovered raised the question of the role of this virus in the pathogenesis of the hemolysis. Suggested mechanisms by which viruses may produce autoimmune hemolytic anemia are by (a) alteration of erythrocyte surface antigens leading to autosensitization [2], (b) eliciting antibody which has cross-specificity with red cell surface antigens [2], (c) acting as an adjuvant [4], (d) autosensitization in the setting of a disturbed immune system [10], or (e) direct effect of viruses on erythrocytes [8, 9].

The implication of viral responsibility in the pathogenesis of autoimmune hemolytic anemia has been chiefly by clinical association [6, 10]. However, in none of these studies has the virus or the other components of the hemolytic system been tested to prove their independent or collective roles in the production of erythrolysis.

The action of autoantibody on the cell membrane in the production of spherocytosis has been described by NEGRINI [5] and DAMESHEK and SCHWARTZ [3], who produced spherocytosis with autoimmune and heteroimmune red cell antibodies. Since the patient's cells incubated with autologous serum were less stable than when incubated with homologous serum, it appears that hemolysis in this patient required the presence of an antibody. Since exhaustive viral studies failed to demonstrate a rise in serum antibodies to poliovirus III, an antibody may have been present which was directed against the red cell-virus union rather than the virus alone, or a faulty antibody directed against the virus may have been present. An immunopathy, although not detected, may be an alternative explanation for the lack of antibody response [10].

The patient's cells incubated without the virus demonstrated an increased fragility compared to control cells. This perhaps suggests an intrinsically defective erythrocyte and could be explained by virus modification of the red cell stroma *in vivo* or defective erythrocyte morphogenesis mediated by the virus. The patient's cells were markedly less stable in the presence of virus than in a virus free system, indicating a direct action of the virus on the red cell membrane may cause accelerated *in vivo* hemolysis. It is possible that the medium in which the virus was suspended may have contributed to the observed osmotic instability. However, such is unlikely since the virus was highly concentrated and the medium was osmotically physiologic.

The patient's cells were more fragile in the presence of exogenous complement than when incubated in the complement free system. The exogenously supplied complement in our system was not independently cytotoxic [7] since the control system does not demonstrate osmotic instability (curve F). Therefore, complement contributed to the instability of the patient's erythrocytes, although it is not clear whether viral action, antibody action or both are complement-dependent.

Maximal hemolysis *in vitro* occurred when the patient's cells were incubated with autologous serum, complement, and poliovirus. Thus, it would appear that in the case studied, an intrinsic red cell defect manifest in the presence of virus, autoantibody and complement is operative in the production of maximal *in vitro* erythrolysis. *In vivo* hemolysis may be an additive phenomenon wherein minimal hemolytic activity occurs when only defective red cells are present and maximal hemolysis may occur when all the components of erythrolysis (autoantibody, complement, virus) are operative.

Acknowledgement. The critical review and helpful suggestions of Dr. G. John Buddingh are gratefully acknowledged. Drs. Larry Hebert and Sheila Moore (*Department of Pediatrics, Earl K. Long Memorial Hospital, Baton Rouge, La.*) provided the clinical material.

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Acta haemat. 53 315–320 (1975)

Further Investigations of the γ-Chain in a Ghanaian Adult, Homozygous for Hereditary Persistence of Fetal Haemoglobin

Isolation of γ CB-3 Peptides and $^{G}\gamma$ $^{A}\gamma$ Ratio Determination in Human Hb F

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Key Words γ-chains of Hb F · Dansyl Edman technique · Fetal haemoglobin · Haemoglobinopathies · Hereditary persistence of Hb F

Abstract. The γ-chain in a Ghanaian homozygous for hereditary persistence of fetal haemoglobin was considered to be of the $^{G}\gamma$ type on the basis of the amino acid analysis of γTp XIV (γ133–144) of the Hb F of this subject [1]. Recently, the sequence of residues γ134–137 of the γ-chain of this subject was determined and found to contain some alanine at position γ136. It is therefore of the $^{G}\gamma + ^{A}\gamma$ type. A rapid technique for the isolation of γCB 3 (γ134–146) peptides in human fetal haemoglobin for $^{G}\gamma$ $^{A}\gamma$ ratio determination is described.

The two types of γ-chain which occur in normal fetal haemoglobin differ at position 136, being glycine in $^{G}\gamma$-chains and alanine in $^{A}\gamma$-chains [2]. These γ-chains are products of non allelic genes which appear to be expressed independently. However, recent studies have shown that several factors such as age and pathological conditions may influence the $^{G}\gamma$-$^{A}\gamma$ ratio. The ratio of γ-chains with glycine at position 136 ($^{G}\gamma$) to those with [illegible]

[illegible]

suppression of the $^{A}\gamma$-chain gene, to no residue of glycine at γ136, i.e. complete suppression of the $^{G}\gamma$-chain gene, and instead, a whole residue of alanine at γ136. Patients of African and Indian origin homozygous for

HPFH have been found to synthesize mainly the $^{G}\gamma$-chain type [3, 4]; whereas those of Caucasian origin have been shown to synthesize mainly the $^{A}\gamma$ type [5]. In our previous publication [1], a patient from Ghana, homozygous for HPFH was reported to synthesize $^{G}\gamma$-chains only, on the basis of the amino acid analysis of γTp XIV (residues 133–144), of the Hb F on this subject. On reading our article, Prof. W. A. SCHROEDER of the California Institute of Technology, Pasadena, wrote: '... when we have had analyses with Gly = 0.91 and Ala = 2.17 such as you show for γTp XIV, it has been shown by PTH degradation of γ CB-3 that the departure of these values from 1 00 and 2 00 was indeed real, both PTH (3-phenyl-2-thiohydantoin)-glycine and PTH-alanine were detectable in position 136.' To test this suggestion, the γ CB-3 fragment was isolated using a technique which had just been developed. Dansyl-Edman degradation of the γ CB-3 fragment revealed DNS-glycine with detectable DNS-alanine at position γ136. The present paper corrects our previous report on the type of the γ-chain in this subject, to read as $^{G}\gamma$ + $^{A}\gamma$ rather than $^{G}\gamma$ only. The present report will also include a description of a rapid technique for the isolation of γ CB-3 peptides for $^{G}\gamma$:$^{A}\gamma$ ratio determination in human fetal haemoglobins.

Materials and Methods

Blood samples were obtained from infants heterozygous for Hb F Texas I [6], Hb F Port Royal [7], Hb F Poole [8] and adult homozygous for HPFH [1], homozygous Hb S with raised Hb F, and a patient with raised Hb F due to leukaemia. The Hb F fractions were either separated from haemolysates by DEAE Sephadex chromatography [9] prior to removing the haem [10] or the total haemolysate was used for the determination of glycine/alanine ratio at position γ136. Each sample was divided into two portions. In the first, chain separation was carried out [11], and the γ-chain digested with trypsin and fingerprints prepared [12]. The γTp XIV (133–144) peptide was separated from γTp II (9–17) by paper electrophoresis at pH 3.5 as previously described [1]. Globin from the other portion (10 mg) was treated with cyanogen bromide [13] in 70% formic acid and lyophilised. It was then re-dissolved in pH 6.4 buffer (pyridine acetic acid water 100 4 900 by volume) and applied on Whatman No 3 papers. Electrophoresis was carried out for both preparations in the same pH-6.4 buffer for 1 h in a Michl type tank at a gradient of 55 V/cm, followed by ascending chromatography (isoamyl alcohol pyridine water 6 6 7 by volume) for 30 h. Peptides on the fingerprints obtained were identified by staining with 0.02% ninhydrin in acetone. The same procedure was carried out on globins from whole haemolysates, except that the starting materials were calculated to contain 10 mg Hb F. In two of the samples, the γCB-3 peptides were eluted with 0.5 M

Table I

	Hb F Port Royal		Hb F Texas I		Hb SS high Hb F		High F/leukaemia	
	γ CB-3	γTp XIV	γ CB-3	γTp XIV	γ CB-3	γTp XIV	γ CB-3	γTp XIV
Threonine	0.91	0.96	0.96	1.01	0.90	0.96	0.92	0.95
Serine	2.53	3.02	2.54	2.90	3.07	3.01	2.50	2.59
Glycine	1.09	1.10	0.09	0.12	0.59	0.56	0.74	0.77
Alanine	2.17	1.98	3.19	3.11	2.52	2.51	2.58	2.56
Valine	2.31	2.21	1.98	2.20	2.17	2.20	2.19	2.20
Methionine	–	0.33	–	0.47	–	0.45	–	0.31
Leucine	1.00	1.00	1.00	0.98	1.00	1.00	1.00	1.00
Tyrosine	0.32	–	0.82	–	0.76	–	0.21	–
Histidine	0.76	–	1.00	–	0.84	–	0.84	–
Arginine	0.92	1.05	0.99	0.96	1.11	0.94	0.73	0.98
Total, nmol, residue	25.44	14.84	10.72	15.10	10.32	14.71	31.99	12.61

NH_4OH lyophilised and the sequence of the first 4 residues determined using the dansyl Edman degradation technique [14]. The dansyl derivatives were chromatographed in polyamide layer plates using the solvents described by WOODS and WANG [15].

Results

The pattern of the cyanogen bromide peptides of Hb F in a fingerprint is shown in figure 1. γ CB 3 (γ134–146) and α CB 2 (α33–76) separate quite neatly from the rest. α CB 1 (α1–32), α CB-3 (α77–141) and γ CB 1 (γ1–55) do not separate under these conditions. When globin from total haemolysate was used the separation of γ CB 3 and α CB 2 was not affected because β CB 1 (β1–55) and β CB 2 (β56–146) remained at the point of origin. The same applied to δ CB 1 (δ1–55) and δ CB-2 (δ56–126). δ CB 3 (δ127–146) is a small peptide, slightly bigger than γ CB 3 and more positively charged than γ CB 3. It would therefore be expected to move fastest towards the negative electrode but this peptide was *not detected presumably due to very low concentrations of Hb A_2* in human haemolysates. The amino acid compositions of γ CB 3 peptides were compared with the amino acid compositions of purified γTP XIV and the glycine alanine ratios were found to be almost identical. On dansyl Edman degradation of the γ CB 3 peptide from Hb F Texas I only

HPFH have been found to synthesize mainly the $^{G}\gamma$-chain type [3, 4], whereas those of Caucasian origin have been shown to synthesize mainly the $^{A}\gamma$ type [5]. In our previous publication [1], a patient from Ghana, homozygous for HPFH was reported to synthesize $^{G}\gamma$-chains only, on the basis of the amino acid analysis of γTp XIV (residues 133–144), of the Hb F on this subject. On reading our article, Prof. W. A. SCHROEDER of the California Institute of Technology, Pasadena, wrote '... when we have had analyses with Gly = 0.91 and Ala = 2.17 such as you show for γTp XIV, it has been shown by PTH degradation of γ CB-3 that the departure of these values from 1.00 and 2.00 was indeed real, both PTH (3 phenyl-2-thiohydantoin) glycine and PTH-alanine were detectable in position 136.' To test this suggestion, the γ CB-3 fragment was isolated using a technique which had just been developed. Dansyl Edman degradation of the γ CB 3 fragment revealed DNS-glycine with detectable DNS-alanine at position γ136. The present paper corrects our previous report on the type of the γ-chain in this subject, to read as $^{G}\gamma$ + $^{A}\gamma$ rather than $^{G}\gamma$ only. The present report will also include a description of a rapid technique for the isolation of γ CB-3 peptides for $^{G}\gamma$:$^{A}\gamma$ ratio determination in human fetal haemoglobins.

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Glycine	1 09	1 10	0 09	0 12	0 59	0 56	0 74	0 77
Alanine	2.17	1 98	3 19	3 11	2.52	2.51	2.58	2.56
Valine	2.31	2.21	1 98	2.20	2 17	2.20	2.19	2.20
Methionine	–	0 33	–	0 47	–	0 45	–	0.31
Leucine	1 00	1 00	1 00	0 98	1 00	1 00	1 00	1 00
Tyrosine	0 32	–	0 82	–	0 76	–	0.21	–
Histidine	0 76	–	1 00	–	0 84	–	0 84	–
Arginine	0 92	1 05	0 99	0 96	1 11	0 94	0 73	0 98
Total, nmol residue	25 44	14 84	10 72	15 10	10 32	14 71	31 99	12.61

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Results

The pattern of the cyanogen bromide peptides of Hb F in a fingerprint is shown in figure 1 γ CB-3 (γ134–146) and α CB-2 (α33–76) separate quite neatly from the rest α CB-1 (α1–32), α CB-3 (α77–141) and γ CB-1 (γ1–55) do not separate under these conditions When globin from total haemolysate was used the separation of γ CB-3 and α CB-2 was not affected because β CB-1 (β1–55) and β CB-2 (β56–146) remained at the point of origin The same applied to δ CB-1 (δ1–55) and δ CB-2 (δ56–126) δ CB-3 (δ127–146) is a small peptide, slightly bigger than γ CB-3 and more positively charged than γ CB-3 It would therefore be expected to move fastest towards the negative electrode, but this peptide was not detected, presumably due to very low concentrations of Hb A_2 in human haemolysates The amino acid compositions of γ CB-3 peptides were compared with the amino acid compositions of purified γTP XIV and the glycine/alanine ratios were found to be almost identical On dansyl Edman degradation of the γ CB-3 peptide from Hb F Texas I only

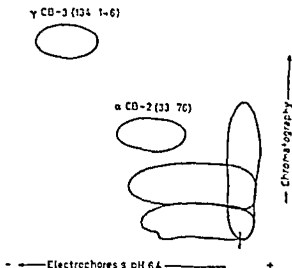


Fig 1 Fingerprint of the soluble cyanogen bromide peptides of Hb F

DNS alanine was found at position 136 with no detectable glycine The γ CB-3 fragment from the HPFH homozygote gave mainly DNS-glycine for position 136 but with a definite trace amount of DNS-alanine No DNS-alanine was detected at positions γ134, γ135 and γ137 As stated already, the nature of the γ-chain in this HPFH case should therefore have been described as $^{G}\gamma + {}^{A}\gamma$ rather than $^{G}\gamma$ only

Discussion

Human haemoglobins have a methionine residue at positions α32 and 76, β55, δ55 and 126, and γ55 and 133 Cyanogen bromide breaks the α-, β-, δ- and γ-chains at these positions to smaller peptides Of these fragments γ CB-3 (134–146) is of particular significance because the duplication of the γ-chain is located at position 136 which can be either glycine or alanine [2], and all known γ-chain mutations involve one of these chains but not both simultaneously

The fragments that are obtained on treatment of Hb F with cyanogen bromide are α CB-1 (32 residues), α CB-2 (44 residues), α CB-3 (65 residues), γ CB-1 (55 residues), γ CB-2 (78 residues) and γ CB-3 (13 residues) Except for α CB-2 and γ CB-1 these peptides can be separated by passage through two 1 × 160 cm columns of Bio-gel P-100 in series which

are developed with 50% acetic acid The γ CB 3 peptide obtained by this method has to be purified further in columns of Dowex 1 [2] The peptides are detected by UV spectrophotometry, and since γ CB 3 peptide has only one tyrosine at position 145, large quantities of starting material are required for easy detection. The other approach involves chain separation of Hb F and tryptic hydrolysis of the γ-chains Peptide chromatograms are prepared and the γTp XIV peptide is isolated and analysed [1, 16] The γTp XIV has to be separated from γTp II by paper electrophoresis in a pH 3.5 buffer (pyridine acetic acid water 5.50 945 by volume) a process which results in loss of material up to 50% Using these methods only few specimens can be handled at a time and relatively large quantities of starting materials are needed The technique described in the present paper provides an efficient, quick and economic way of achieving the same end

In the γ CB 3 peptide, the values of glycine and alanine are 1 00 and 2 00 for the $^{G}\gamma$-chain when the $^{A}\gamma$-gene is completely suppressed and 0 00 and 3 00 for the $^{A}\gamma$ when the $^{G}\gamma$-gene is completely suppressed Variations from these values even at the level of 0 1 residue may indicate that neither gene is completely nonfunctional It had been our custum to ignore such small variations in molar ratios The present study however, shows their significance

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ferrin molecules has been found non-existent or minimal [18, 21]. AISEN and LEIBMAN [2] demonstrated radioiron transfer between electrophoretically separable transferrins in the presence of citrate. Stimulation for the present study came from FLETCHER and HUEHNS's [8] experiments demonstrating different rates of iron delivery to reticulocytes from the two transferrin iron binding sites *in vitro*.

Material and Methods

Preparation of transferrin solutions. The procedures followed have already been described in detail [12]. In some experiments to the saline transferrin solutions 10^{-2} M Na citrate dissolved in saline was added to give final citrate concentrations of 10^{-3} – 10^{-4} M.

Preparation of rabbit reticulocytes. 3 kg rabbits of different origin were bled three times from the ear vein up to 1% of body weight at 2-day intervals. 2 days following the last bleeding blood was drawn by cardiac puncture and immediately cooled to 0 °C. At that time the haematocrits were 25–35%, and the reticulocyte counts ranged between 10 and 25%. The cells were washed twice with cold saline and the packed cells, referred to as packed reticulocytes, used for incubation studies. The haematocrit of the packed cells was 65–80%.

Conditioning of transferrin solutions. 80–90% iron saturated transferrin solutions in saline (0.5 mg transferrin/10 ml, pH 7.4) were exposed to packed reticulocytes in volume ratios of 1:2.5–1:4 in different experiments. After 20 min of incubation at 37 °C the supernatant was recovered by sharp centrifugation at 0 °C. The concentration change of transferrin due to the saline content of the packed reticulocytes was corrected on the basis of volumes and haematocrits. Changes in ^{59}Fe transferrin saturation were monitored by radioactivity measurements of the solution prior to and after incubation. Generally within 20 min 50% of the radioiron initially present had been taken up by cells. The method just described is similar to the procedure originally introduced by FLETCHER and HUEHNS [8]. Preparations conditioned in this way are referred to as preincubated solutions.

In vitro studies. Four different transferrin solutions identical in protein concentration and ^{59}Fe saturation were simultaneously investigated in respect to their capacity to deliver iron to reticulocytes: (a) preincubated, (b) freshly prepared, (c) preincubated containing 10^{-3} M citric acid, and (d) freshly prepared containing citrate (10^{-3}–10^{-4} M). The solutions were kept at 37 °C throughout the experiment in a shaking bath, the pH being continuously checked. At 1 to 2 hour-intervals, 0.2 ml portions were removed and incubated with 0.5 ml of packed reticulocytes for 20 min at 37 °C. Radioiron removal from the transferrin solution was measured after rapid cooling to 0 °C followed by centrifugation.

Studies in rats. Female Wistar rats, weighing 250–300 g, were made slightly anemic by cardiac puncture 2 days prior to the experiment. The amount of blood removed was 1.5% of body weight. Two types of transferrin solutions employed for

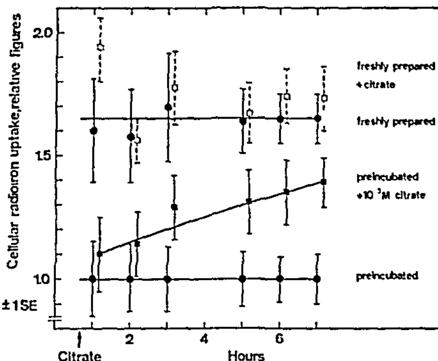


Fig 1 Relative iron uptake by reticulocytes from preincubated transferrin containing citrate and freshly prepared transferrin as compared to the preincubated citrate free preparation (5 experiments) The solutions were incubated at 37 °C for 7 h and samples removed at the time points indicated for a 20-min exposure to packed reticulocytes The radioiron incorporation for the citrate free preincubate was 30–50% in different experiments The amount of citrate added to the freshly prepared solutions (open symbols 13 experiments) varied between 10^{-3} and 10^{-2} M the results were pooled because no concentration-dependent effect was evident

cirate free solutions was reduced by 50% Addition of citrate to freshly prepared solutions failed to modify iron uptake In preliminary experiments it could be shown that the concentration of citrate influenced the rate of iron transfer from preincubated transferrin solutions to reticulocytes whereas no such influence could be detected in freshly prepared solutions

Studies in rats The radioiron plasma disappearance was significantly more rapid if the ^{59}Fe had been added after *in vitro* reticulocyte exposure The average t $^1/_2$ for the two groups were 49 0 and 84 4 min, respectively ($p<0.05$) No differences in mean serum iron levels and haematocrits were observed (table I)

Discussion

FLETCHER and HUEHNS [8] reported that a radioiron transferrin solution which had been preincubated with immature red cells delivered significantly less radioactivity to reticulocytes than a fresh preparation equal in radioiron and transferrin concentration. This difference persisted if both solutions were fully saturated with cold iron prior to the final reticulocyte exposure. Preferential iron uptake from one of the two binding sites by erythroid cells was concluded. This would imply dissimilar behaviour of binding sites, a concept compatible with the partly non repeating amino acid sequence of the molecule [15, 22] made most likely by paramagnetic resonance spectroscopy of the chromium and the iron transferrin complex [1, 3, 19], and by the binding characteristics of lanthanide ions to transferrin [16].

The above *in vitro* observation has been confirmed by CHERNELCH and BROWN [5], and once more in the studies reported herein. However, if citrate was added to the preincubated transferrin in physiological concentration the availability of iron for reticulocytes gradually increased. Whereas, during a 7 hour period the fresh solution exceeded the preincubate in iron donating capacity by a constant factor of 1.65, this difference was halved after 5 h in presence of citrate in the preincubated solution. No effect of citrate on the fresh solutions was evident. As mentioned earlier, citrate mediated iron exchange between different transferrin species has already been demonstrated [2]. The present finding indicates that citrate also promotes an interbinding site iron exchange. Using a different *in vitro* system, intraplasmatic iron redistribution among transferrin molecules, curtailed by removal of low molecular compounds through dialysis but restored by citrate, has recently been confirmed in this laboratory [10].

The significance of chelating agents in general and of citrate in particular in iron metabolism has been stressed by SALTMAN [20]. Citrate removes iron from transferrin in greater amounts than expected from the stability constant [6] and the transfer of ferric iron from citrate to apotransferrin in an isomolar solution is a slow process terminated after 20 h only [4]. The rate limiting step appears to be the formation of the ternary complex citrate ferric iron transferrin which is broken down very rapidly. The transitional character of such a compound is supported by the minimal changes of spectral absorbancy induced by 10^{-3} M citrate added to a 10^{-4} M solution of iron-saturated transferrin [2]. Chelating agents other

than citrate might contribute to intraplasmatic iron exchange Such a role may be visualized for some amino acids and sugars keeping iron soluble in the alkaline duodenum [4], and for several phosphate compounds apparently involved in iron transfer between transferrin and ferritin [17]

Interbinding site iron transfer still appeared to be rather slow *in vivo* as evident from persistent differences in plasma clearance rates of ^{59}Fe bound to different transferrin preparations Briefly, slightly anemic rats obtained one of two transferrin solutions which were first saturated with ^{59}Fe or ^{55}Fe, and after reticulocyte exposure, resaturated with the opposite iron isotope As expected the ^{59}Fe added after *in vitro* incubation disappeared more rapidly from circulation being preferentially attached to the reticulocyte-oriented binding sites Rapid iron redistribution among binding sites probably would have caused a progressive similarity in clearance rates Nevertheless, these results confirm the functional non equivalence of the two transferrin iron binding sites in an *in vivo* system Exactly the opposite phenomenon was encountered in polycythemic rats injected with similar transferrin preparations [11] Radioiron was cleared more rapidly from the compound initially labeled, and preferentially accumulated in the liver Thus, through minimizing iron flow to the erythropoietic tissue a liver-oriented transferrin binding site became apparent

The present investigations are open to a number of possible criticisms It must be conceded, for instance, that the biological significance of interbinding site iron exchange is by no means clarified If it proceeds at the rate observed in our rather simple *in vitro* system, it would be difficult to recognize an important physiological process Furthermore a system including human transferrin, rabbit reticulocytes, and rats may introduce variables little related to physiology This object creates little concern in view of the rates of reticulocyte iron uptakes and plasma iron clearances as measured in these studies, being entirely in the order of magnitude found in a homogenous animal system [9] Finally, one might concede limited significance to data based on the use of purified transferrin possibly denatured to some extent or deprived from hypothetical molecules essential for physiological function

The above reservations in mind we conclude that *in vivo* evidence for a functionally heterogeneous transport iron pool was obtained The iron distribution between transferrin binding sites and, independently, the saturation degree of the individual carrier proteins, are intimately linked to erythropoietic activity In addition iron exchange between binding sites possibly imparts an internal dynamic to the transport iron compartment

It is attractive to envisage a decisive function of this complex system for iron distribution following specific tissue demands. The hypothesis has been advanced that iron absorption from the gastrointestinal tract and iron mobilization from storage sites is governed by the number of unoccupied haemoglobin-oriented transferrin binding sites [8]. Our findings are compatible with this idea. From a practical point of view, reevaluation of ferrokinetic data seems compelling: specifically, the slow components of plasma radioiron disappearance may not only be caused by reflux phenomena [7], but also mirror iron fractions leaving the plasma compartment at a slow rate.

Acknowledgements. This investigation was supported by grants from the Schweizerischer Nationalfonds zur Förderung der wissenschaftlichen Forschung (3.89.69, 3.393.70 and 3.7710.72) and the Hartmann Müller Stiftung der Universität Zürich.

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Acta haemat 53 329-335 (1975)

Binding of Folic Acid to Serum Proteins

V. Tritiated Folic Acid in the Liver

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Key Words Carrier proteins Folic acid metabolism Gel chromatography · Liver cell metabolism

Abstract Sephadex G-200 chromatographies of the liver cell sap of rabbits, 1, 3 and 6 days after an injection of ^3H folic acid activity (FAA) into the circulation were used in an attempt to study the binding of this labelled FAA to liver proteins. The labelled FAA was quickly accumulated in the liver, and in the cell sap it was grouped chromatographically to two maxima, which corresponded to the two maxima of microbiological FAA (*L. casei*) The maxima were eluated in the chromatography together with cell sap proteins. The possible coupling of FAA to proteins in the liver is discussed

In vitro, about half of the serum folic acid activity (FAA) is eluated together with proteins from the DEAE Sephadex A-50 column [5] This half forms 3 FAA maxima in the protein area [6] More detailed chromatographic study has shown that these maxima are projected to the α_2-macroglobulin, transferrin and albumin areas [7, 8] quantitatively in this order In healthy test subjects the maxima are similar, though the FAA of the transferrin area lies at a slightly higher level in women than in men and the distribution in women varies slightly according to the different phases of the menstrual cycle [14] When pregnancy develops, the FAA distribution undergoes a distinct change [9] and the use of oral contraceptives has much the same effect [14] Diphenylhydantoin increases the binding of FAA to the transferrin zone [10] Certain diseases such as hyperthyroidism myeloma, pernicious anaemia and liver diseases clearly affect the FAA distribution [11]

The FAA levels of the liver are high and the liver is, as far as is known the metabolic centre of folic acid. In the present study an effort

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The FAA levels of the liver are high, and the liver is, as far as is known, the metabolic centre of folic acid. In the present study an effort

was made to observe the metabolism of tritium labelled folic acid in the liver, giving special attention to the way in which FAA seemed to be coupled to liver proteins in the course of this process

Material and Methods

Folic acid dissolved in physiological saline solution was injected intravenously into the rabbit s ear (folic acid 3 5 9 ^{3}H(n) potassium salt Radiochemical Centre Amersham specific activity 31 Ci/mmol) The rabbit which was followed up for 24 h was given 70 μCi the one followed up for 3 days 100 μCi and the animals followed up for 6 days 45 μCi After the injection the test animals stayed in their cages on the normal dietary regime for the relevant 1 3 and 6 days respectively

The livers of the rabbits were removed carefully rinsed with saline solution and cooled in an ice bath Approximately 10 g of the liver was sectioned and homogenized in 100 ml physiological saline in a Potter Elvehjem homogenizer (with a teflon piston) The suspension obtained was further treated in an ice bath with an ultrasound equipment (MSE ultrasonator 1 5 A) for 3 min and finally centrifuged for 30 min at 14 000 rpm

The supernatant (40 ml) of the liver suspension was then fractionated immediately in column chromatography (Sephadex G 200 Pharmacia Uppsala) using techniques described earlier [6] In each chromatography 180 samples (10 ml) were collected and the ^{3}H activity as well as the microbiological FAA of every sample were measured The rate of elution was 45–60 ml h

The proteins of both the supernatant of the liver suspension and its chromatography fractions were determined with the biuret method and the folic acid with the *L casei* method (after the addition of ascorbic acid) as reported earlier [6]

Samples (0.2 ml) of the supernatant of the liver suspension and its chromatography fractions were taken into scintillation flasks for the determination of ^{3}H activity The ^{3}H activity of the serum of the test animal was determined in the same way After this scintillation fluid (10 ml Merck) was added and the ^{3}H activity was measured for 10 min per sample with a Packard Tri Carb fluid scintillation counter (Model 3375)

The results are presented in fig 1 and table I

Results

Figure 1B shows that the ^{3}H FAA injected into the test animal s vein is seen in the chromatogram of liver cell sap concentrated primarily into two maxima coinciding with the site of the microbiological FAA analysed simultaneously with *L casei* (fig 1A) A small proportion of the latter of course, is ^{3}H FAA injected into the vein

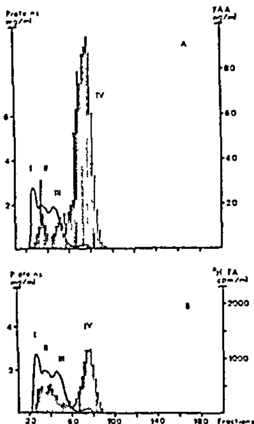


Fig. 1. A Typical chromatography curve of the cell sap of rabbit liver 24 h after intravenous injection of ^{3}H-FAA. The continuous line indicates protein areas I–IV and is identical to that in *B*. Vertical shading indicates the microbiological FAA (*L. casei*). Note the two distinct maxima in the protein area. *B* The continuous line synchronously with the former indicates protein areas I–IV. Vertical shading indicates ^{3}H-FAA. Note the two distinct maxima coinciding with those of the microbiological FAA in the protein area.

Both the radioactive and microbiological FAA are eluted from the column in Sephadex G-200 chromatography simultaneously with proteins. When this gel is used, the proteins form 4 groups (protein areas I-IV, fig. 1A, B). These protein groups are not sharply differentiated. After the elution of proteins, chromatography was still prolonged up to

Table I

Liver cell sap	Animal		
	1	3	6
Proteins, mg/ml	17 9	14 2	14 5
Recovery of injected ^{3}H FAA, %	31	56	32
Activity of ^{3}H FAA in protein areas %	81	78	76
^{3}H FAA/mg proteins, cpm/mg	450	1 039	408
^{3}H FAA in protein areas I III, %	40	50	27
^{3}H FAA in protein area IV, %	60	50	73
IV/I-III	1 48	1 04	2.78
Serum			
^{3}H FAA, cpm ml	594	0	223

^{3}H FAA was injected into the veins of three rabbits A Sephadex G 200 chromatography of the liver cell sap was carried out for one animal after 24 h (1) for the second after 3 days (3) and for the third after 6 days (6) Recovery indicates the amount of the injected ^{3}H FAA in % recorded in the animal s liver after these periods. cpm = Counts per minute Protein areas I-IV are shown in fig 1A and 1B The serum ^{3}H FAA of each animal recorded at the same time as the liver sample was studied is shown at the bottom of the table

the 180th fraction, but neither radioactive nor microbiological *(L. casei)* activity was any longer eluated from the column (fig 1A B) The results after 1, 3 and 6 follow-up days are qualitatively similar, in other words the FAA maxima coincide with those of the protein chromatogram

Table I reveals, however, that when more than 3 days have passed since the injection of ^{3}H-FAA, changes begin to occur in the ^{3}H FAA distributions of liver cell sap activity diminishes in protein areas I-III and increases in protein area IV At the same time, ^{3}H activity in clearly measurable quantities again begins to appear in the serum of the test animals This activity at the 3-day follow up was 0 cpm/ml (table I) With prolongation of the follow-up period, a slight tendency towards a diminishing percentage of the ^{3}H FAA eluated with proteins into the liver cell sap is observed

Discussion

A considerable amount of the tritium labelled FAA injected into test animals' circulation seems to accumulate in the liver There, in the liver cell sap, it is grouped into two maxima These maxima are eluated to-

gether with proteins which, in turn are distributed into 4 protein areas in Sephadex G 200 chromatography The microbiological FAA (*L casei*) is also eluated with the said proteins, and the maxima coincide with the two ^{3}H FAA maxima mentioned The first of the two radioactive maxima to be eluated diminishes relatively with time, while the second grows At the same time ^{3}H FAA appears in the serum.

Relatively little is a yet known of the binding relations of folic acid to serum proteins However, it is known that at least half of the total serum FAA is eluated in chromatography with the proteins, and in the electrophoretic study of the various protein fractions FAA moves in the manner peculiar to each protein [12] while the unbound FAA follows a separate course [unpublished observation] Transferrin, α_2-macroglobulin and albumin have been suggested as the FAA carrier proteins [12] At least for transferrin, findings similar to ours have been reported by certain other authors [15], but a differing view has also been published [3] In different animal species the binding proportions seem to be different [4 13]

It is highly presumable that the coupling of FAA to proteins takes place in the liver, side by side with FAA metabolism in general The present study demonstrated in fact, that liver cell sap chromatography has two separate FAA maxima and that these maxima are eluated together with apparently, fairly large molecular proteins The question arises whether the labelled FAA is really attached to these proteins or whether the FAA for some other reason is eluated together with the proteins One and perhaps the most important, reason is that the molecular weight of the possibly polymerized FAA would be equal to those of the eluated proteins However, the FAA eluated in the first protein maximum can hardly be of a molecular weight as high as the usually very large molecular protein that is eluated in this maximum

The FAA metabolism in the liver has been studied also from aspects other than binding to proteins It has been noted that some 85% of the FAA in the liver are in the form of reduced pteroyl polyglutamates (more than 3 glutamate moieties/molecule), 12–15% in the form of reduced pteroyl glutamate, ca 1% in the form of pteroyl-diglutamate and only traces in the form of reduced pteroyl monoglutamate [1] The same authors found that endogenous and injected ^{3}H FAA is concentrated in liver mitochondria and the cell-sap fraction (21 and 71%, respectively) [2] They also pointed out that the binding of FAA to liver proteins is not known [2]

We began an electrophoretic analysis of the fractions obtained by chromatography but found that the protein concentrations were too small to obtain a good immunoelectrophoresis When we tried a simultaneous autoradiography and immunoelectrophoresis, it was found that the ^{3}H FAA radiation was not strong enough to show on even a sensitive film Hence the direct and conclusive evidence of the binding of ^{3}H FAA to the liver cell sap proteins could not be verified by the present methods Only the eluation simultaneously with proteins justifies the *assumption* that FAA probably is bound to proteins in the liver and only then passes into circulation where it occurs at least partly bound to proteins

A great deal of additional study, preceded by advances in chromatographic techniques is required before the present laborious problem can really be tackled in greater detail

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Acta haemat 53 336-340 (1975)

Quantitative Determination of Immunoglobulins in Chronic Renal Failure

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Key Words Blood urea Immunoglobulins Proteinuria Renal failure Uremia

Abstract Immunoglobulins IgG, IgM and IgA in the serum of 61 patients suffering from chronic renal failure of various etiology and not being under hemodialysis treatment were measured Cases of nephropathy, due to collagen or blood disease or uremia with heavy proteinuria were excluded No statistically significant difference was found between patients and controls in respect to the serum level of the 3 immunoglobulins studied No correlation was found between the level of serum immunoglobulins and the blood urea or the amount of protein daily excreted in the urine

Uremic patients show an abnormal immune response [2, 4, 6, 7, 9, 11, 13, 14] The alterations in the globulin fraction by changes in the immunoglobulins may be a contributory factor On the other hand, the use of immunosuppressive drugs for treatment of the renal disease or for protection from rejection episodes after renal allografting needs a good estimation of the immunological state of the patients Proteinuria is a characteristic feature of renal insufficiency, and it has been established that proteinuria itself can result in a catabolic hypogammaglobulinemia with a reduction in the IgG fraction [1] The present study concerns itself with the quantitative determination of the 3 major serum immunoglobulins (IgG, IgM and IgA) and with their correlation to blood urea or the daily amount of protein excreted in the urine

Material and Methods

89 adults of both sexes, aged between 20 and 70 years, were studied They were divided into two groups.

Table I. Blood serum immunoglobulin levels (mg/100 ml) in 61 uremic patients and 28 normal controls. Mean ± SD (range)

Immunoglobulins	Uremic patients	Normal controls	Significance[1]
IgA	264 ± 142 (90 – 900)	293 ± 93 (85 – 440)	n.s.
IgG	1,127 ± 516 (250 – 2,800)	1,312 ± 596 (500 – 2,000)	n.s.
IgM	89 ± 45 (27 – 270)	100 ± 37 (34 – 170)	n.s.

[1] Student's t test; n.s. = Not significant.

Group I. 61 uremic patients suffering from chronic renal failure of various etiology with blood urea values between 54 and 450 mg%, and protein excreted between 0 and 5.25 g/24 h. In most cases chronic glomerulonephritis, chronic pyelonephritis and polycystic disease has been diagnosed, while in a few cases it was not possible to identify the exact etiology of the disease. Patients with chronic renal failure due to collagen or blood diseases, as well as those with heavy proteinuria have been excluded. No patient had undergone peritoneal dialysis or hemodialysis.

Group II. 28 normal adults were used as controls. IgG, IgA and IgM were quantitated by a single radial immunodiffusion method [10], with the use of commercially available antibody-agar plates (Immunoplates, Hyland Laboratories, Los Angeles, Calif.).

Blood urea was determined by the method of LEVINE *et al.* [8] and urine protein by the method of GOODWIN [3].

Results

The results are shown in table I and in figures 1–3.

Discussion

SCHULTRELLS *et al.* [12], in a study of 45 patients with chronic renal failure, found that in the majority of cases the concentration of immunoglobulins was abnormal, i.e. there was a decrease of IgG in renal insufficiency of short duration and of IgM in chronic cases. LO GRIPPO *et al.* [7] found the following quantitative differences of serum immunoglobulin in 25 cases: 6 patients had decreased IgG, while 2 had an increase, 8 patients had increased IgA and 5 had decreased values, 6 patients had decreased IgM and 3 had an

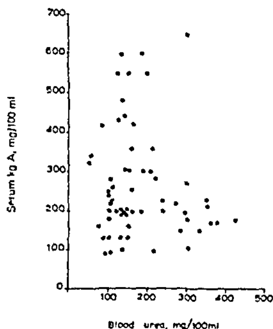


Fig. 1. Relation of serum IgA to blood urea. $r = -0.218$; p = not significant; $n = 61$.

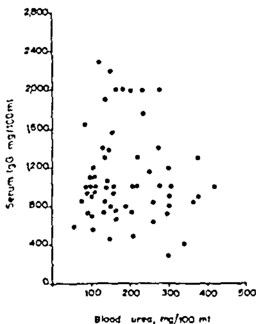


Fig. 2. Relation of serum IgG to blood urea. $r = -0.419$, p = not significant, $n = 61$

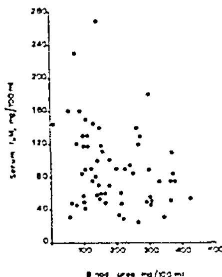


Fig. 3. Relation of serum IgM to blood urea. $r = -0.23$; p = not significant; $n = 61$.

increase, 2 patients registered low levels of all three immunoglobulins, whereas 6 patients had normal levels of all three. According to HOBBS [5] the uremic environment of chronic renal failure is considered to effect a toxic inhibition of globulin synthesis affecting primary IgM, then IgA and finally IgG.

Our findings did not prove any statistically significant difference of immunoglobulins between uremic patients and normal controls. No correlation could be found between the levels of IgG, IgA, IgM and the severity of uremia, as judged by blood urea. In patients with chronic renal disease in a nephrotic stage there may be a significant urinary loss of IgG and to a lesser degree of IgM. Our study concerned with uremic patients without excessive urine protein loss (proteinuria up to 5.25 g/24 h), and it did not prove any correlation to the amount of serum immunoglobulins.

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Acta haemat. 53: 341-346 (1975)

Changes of Trace Minerals (Serum Iron, Zinc, Copper and Magnesium) in Thalassemia

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Key Words. Thalassemia · Trace minerals · Zinc in serum

Abstract. We have determinated serum Zn, Fe, Cu and Mg in 42 patients aging from 3 months to 22 years with homozygous β-thalassemia and thalassemia intermedia and in 36 control subjects of the same ages. Serum zinc was significantly decreased. Serum copper and iron were increased, but magnesium was found to be at normal levels.

The thalassemia syndromes are characterized by an inherited defect in the synthesis of one or more of the peptide chains of hemoglobin [1]. Biochemical changes, especially of the essential trace minerals such as zinc, copper, and magnesium have not been fully investigated in homozygous thalassemia [1, 2]. Clinicians have noticed growth retardation in patients with homozygous thalassemia. Zinc is an essential element, and its deficiency retards growth and maturation in plants, animals and man. A hypogonadal type of dwarfism prevalent in Iran, Egypt and Turkey is found to be related to zinc [3-5]. Therefore, we wanted to study changes of the essential trace minerals zinc, copper, iron and magnesium in thalassemia.

Materials and Methods

A total of 42 cases (39 with homozygous β-thalassemia and 3 with thalassemia intermedia) aged between 3 months and 22 years (17 girls and 25 boys) were studied. 36 subjects of the same ages were used as controls. All of the 42 patients were hospitalized and studied clinically, hematologically and genetically.

Routine hematologic tests were performed with standard methods. Furthermore, the following studies were carried out: hemoglobin A_2 was measured by starch block electro-

Table I. Trace mineral concentrations in thalassemia (biochemical methods)

Minerals in serum	Thalassemia			Controls			p
	n	$\bar{x}$	$\pm s_{\bar{x}}$	n	$\bar{x}$	$\pm s_{\bar{x}}$	
Fe, µg%	19	186.10	9.1	10	98.75	7.2	<0.05
Zn, µg%	16	86.96	4.2	10	176.8	12.6	<0.05
Cu, µg%	19	168.57	3.7	14	128.1	11.4	<0.05
Mg, mg%	14	1.69	0.05	10	2.11	0.43	>0.05

Table II. Trace mineral concentrations in thalassemia (atomic absorption spectrophotometry)

Minerals in serum	Thalassemia			Controls			p
	n	$\bar{x}$	$\pm s_{\bar{x}}$	n	$\bar{x}$	$\pm s_{\bar{x}}$	
Fe, µg%	18	197.06	17.4	22	99.32	5.5	<0.01
Zn, µg%	21	94.70	5.3	22	111.95	5.0	<0.05
Cu, µg%	20	247.80	21.0	22	136.73	4.1	<0.01
Mg, mg%	12	2.34	0.16	22	2.33	0.09	>0.05

phoresis [6] and fetal hemoglobin by alkali denaturation by the method of SINGER *et al.* [7]. In a first group of 20 patients, the levels of iron, zinc, copper and magnesium in serum, were determined according to previously described biochemical methods [5], in the other 22 patients by Perkin Elmer atomic absorption spectrophotometer Model 103 [8, 9].

Results

Table I shows the results obtained in the first group. An increase in serum iron and copper was observed, but serum zinc showed a statistically significant ($p < 0.05$) decrease as compared to the controls. Serum magnesium was found to be normal. Table II shows the results of these determinations in the second group. There were significant increases in serum iron and copper but a significant decrease in serum zinc as compared to the controls. Serum magnesium was again found to be normal.

Discussion

As reported by many authors, serum iron is increased in thalassemia because of inability to use adequate quantities of iron in the production

of hemoglobin, frequent blood transfusions and increased intestinal absorption [1, 11, 12] In our cases, serum iron was found high and the unsaturated iron binding capacity was decreased

Zinc is considered to be an essential trace mineral for growth of plants, animals and man This element has been observed to be an integral part of several metalloenzymes including carbonic anhydrase, alkaline phosphatase, pancreatic carboxypeptidase and lactic dehydrogenase. It is also required for synthesis of nucleic acids and protein metabolism [13–16]

Zinc deficiency was once thought impossible in man owing to the widespread presence of the element in food and water This concept is now changed Zinc deficiency has been found in malnourished populations as well as associated with various dieseases. Human zinc deficiency associated with nutritional dwarfism has been reported for the first time in Iran and Egypt [3, 4]

The principal manifestations of zinc deficiency in man are growth retardation, hypogonadism, decrease in the production of endogenous ACTH, increase to insulin sensitivity disturbance in protein synthesis, and delay in healing of wounds, etc [18]. It has been shown that these clinical manifestations improve after zinc treatment [17]

Several factors may be responsible for zinc deficiency in man, but we consider the following three to be the most important ones

1 Unavailability of zinc Inability of the organism to use the zinc consumed by the diet Phytates, found in large quantities in certain foods, namely cereals, bind zinc and form unabsorbable complexes In addition, calcium, cadmium, phosphate, and chelating agents also affect the zinc availability [15, 17].

2 Loss of zinc The zinc concentration in red blood cells is approximately 12 times that found in plasma, and therefore chronic blood loss causes zinc deficiency According to some reports, schistosomiasis and ancylostomiasis are responsible for zinc deficiency in the Delta region in Egypt The zinc content of sweat was found to be 115 μg ± 30%. Thus, hot climate may cause a loss of zinc up to 2 3 mg/day Consequently, loss of zinc with sweat may be another important factor [17]

3 Miscellaneous factors There have been sporadic reports of low circulating zinc levels in clinical disorders such as liver disease, pernicious anemia, thalassemia, malignant diseases, acute and chronic infections, and myocardial infarction [17]

In the light of these informations, the question arises why the plasma zinc level decreases in thalassemia In the 11 thalassemia patients from Iran investigated by Prasad *et al* [18] a decrease in plasma zinc levels in ad

dition to growth retardation was found The results in our patients are similar With one exception, the patients showed growth retardation as well

As we mentioned above, nutritional factors may be responsible for zinc deficiency in man Most of our cases were from good socioeconomic level and their diets contained adequate amounts of animal proteins None had schistosomiasis and hookworm infections which may cause blood loss Therefore, zinc deficiency in our cases cannot be explained on the basis of blood loss and malnutrition

VALLEE *et al* [19] investigated zinc metabolism in liver dysfunctions and reported zinc deficiency in liver cirrhosis There was no clinical and laboratory evidence of cirrhosis in our patients

Although the explanation of zinc deficiency in thalassemia cannot definitely be made, the symptoms of the deficiency, such as severe growth retardation, bone changes, and retardation in sexual maturation were obvious in our cases Growth retardation was present in all but one Hypogenitalism and sexual retardation were very prominent, especially in two cases of the first group where the serum zinc level was also very low

Our study has demonstrated increased iron and copper levels in the patient s serum ERLANDSON *et al* [20, 21] also found increased serum iron and copper and decreased serum magnesium in thalassemia patients Iron absorption needs copper containing enzymes and cofactors In addition, copper affects release of iron from body stores and utilization of iron in hemoglobin synthesis In the study of PRASAD *et al* [18] serum copper levels were increased in thalassemia Hypercupremia occurs in pregnancy, prematurity, acute and chronic infections iron deficiency anemia, hyperthyroidism and hemochromatosis [22] Hemochromatosis is a principal complication of thalassemia Frequent blood transfusions, increased iron absorption and hemolysis facilitate marked accumulation and excess deposition of iron in tissues causing visceral hemochromatosis

Serum magnesium levels were normal in our patients According to some investigators, serum magnesium is normal [18] but other studies showed abnormally low concentrations in the majority of thalassemia patients older than 15 years [20 21]

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therefore be established conclusively, but the overall ratio of $^{G}\gamma$ to $^{A}\gamma$ chains was determined by analysing the CB-3 peptide [13] and was found to be 2 1 1 for position γ136. As the $^{A}\gamma$-chains amounted therefore to 29% of all the γ-chains and the mutant γ-chain (see above) to 25%, it is most unlikely that the latter would be an $^{A}\gamma$-chain leaving only 4% for the normal $^{A}\gamma$-chain. It is much more likely that the mutant amounts to 36% of the $^{G}\gamma$-chain. The distribution would then be: 71% $^{G}\gamma$ of which about one-third would be the mutant $^{G}\gamma$, and 29% $^{A}\gamma$.

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Acta haemat 53 356–361 (1975)

Chronic Eosinophilic Leukemia Complicated by Epidural Myeloblastoma

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Key Words Eosinophilic leukemia · Myeloblastoma · Transcobalamins · Vitamin B_{12}

Abstract Epidural myeloblastoma which compressed the spinal cord was the first evidence for chronic granulocytic leukemia, eosinophilic type, Ph chromosome negative. This manifestation was preceded by 3 years follow up of a patient with persistent eosinophilia of 60% mature eosinophils. The only clues for the diagnosis of leukemia were splenomegaly and high serum vitamin B_{12}, most of which was bound to transcobalamin I. The latter finding presents a useful diagnostic criterium in myeloproliferative disorders.

Eosinophilic leukemia [1] is a variant of chronic granulocytic leukemia, Philadelphia (Ph) chromosome positive [2–4], or negative [5–7]. Mature cell eosinophilic leukemia with an acute or chronic course and leukemia beginning with increased blast cells in the peripheral blood and/or bone marrow were described [8, 9]. Eosinophilic granulocytes can form green tumors (chloroma) and non-green tumors (myeloblastoma) [10, 11].

In the case to be described the diagnosis of chronic eosinophilic leukemia was made on the basis of the highly increased serum vitamin B_{12} concentration and the corresponding elevation of transcobalamin (TC) I, findings similar to those present in chronic myeloid leukemia [12]. The development of myeloblastoma and subsequently acute granulocytic leukemia confirmed this diagnosis.

Table I

Date	Serum B_{12} pg/ml total	Serum B_{12} pg/ml bound	UBBC pg/ml	TC I %	TC II %	TBC TC I pg/ml	TBC TC II pg/ml	WBC/µl
27.7.73	8,000	8,000	4,000	99	1	7,200	800	14,000
4.2.74	6,800	6,800	3,500	91	9	6,120	680	7,000
16.6.74	4,000	4,000	2,400	80	20	3,200	800	12,900
22.7.74	6,000	3,200	845	73	27	1,500	1,400	4,900
21.8.74	3,200	875	1,000	56	44	912	1,023	2,000
Normal values	500–800	450–750	800–1,100	18–28	70–80	200–300	400–700	4,000–10,000

UBBC = Unsaturated B_{12} binding capacity, TC I = transcobalamin I, TC II = transcobalamin II, TBC = total binding capacity, WBC = white blood cells.

Case Report

H.A., a 42-year-old male, was first hospitalized in July, 1973. Two years previously he underwent thoracotomy because of a pleural effusion with empyema. Eosinophilia and splenomegaly were noticed. On admission there was no history of fever, night sweats or pruritus. Physical examination revealed good nutritional state. The blood pressure was 130/80 mm, regular pulse 80/min. No lymph nodes were palpable. The abdomen was soft and the liver not palpable. A firm spleen was palpable 3 cm below the left costal margin. No abnormal neurological findings. Sedimentation rate (Westergren) 5/15 mm. Hemoglobin 14.1 g%. White cell count 14,000/µl with 63% of mature eosinophils, 15% neutrophils, 2% band forms, 1% basophil, 16% lymphocytes, 2% monocytes, 1% myelocytes. Total eosinophil count 7,400/µl. Platelets 110,000/µl [13]. Blood glucose, urea, electrolytes and liver function tests within normal limits. Repeated stool examinations for parasites negative.

The chest X-ray did not reveal any masses in the lungs or mediastinum. A liver scan showed a normal liver without filling defects and an enlarged spleen. A liver biopsy at this stage revealed normal liver tissue. Sternal aspiration revealed cellular marrow with myeloid/erythroid ratio 4:1. The granulocytic series included 40% eosinophils in different stages of maturation (promyelocytes, myelocytes, metamyelocytes, band forms and mature polymorphonuclears). No other abnormalities were found. Leukocyte alkaline phosphatase 154 (normal 15–75). Ph chromosome was negative [14]. Excessively high levels of serum vitamin B_{12} were found [15]. Vitamin B_{12} binding capacity was very high and most of the vitamin was bound to TC I (table I). The patient was observed for a period of 1 year during which time his general condition was unchanged.

He was hospitalized again 1 year later, in June 1974, with radicular pain and hyperesthesia along the intercostal nerves 9–12. A rapid progression of the neuro-



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ology



Fig. 3. Imprint of the tumor showing 3 blasts. May-Grunwald-Giemsa × 1,000

logical signs occurred with the development of spastic paraparesis and urinary incontinence. On myelography complete obstruction was found at the level of D9. Laminectomy was performed and an epidural vascular greyish-yellow tumor was removed. Sections and imprints revealed a tumor consisting of mature eosinophils, eosinophilic myelocytes and blast cells, a picture compatible with myeloblastoma (Fig. 1-3). No neurological improvement was obtained after removal of the tumor and the spinal column was irradiated with a total dose of 2,400 rad. This therapy did not improve the neurological signs. Pancytopenia was found subsequently. Bone marrow aspiration at this stage showed hypercellular marrow with myeloid-erythroid ratio of 20:1 due to granulocytic hyperplasia. Almost all the cells were blasts and promyelocytes. No Auer bodies were found.

The general condition of the patient deteriorated rapidly with clinical signs of septicemia with blood cultures positive for *E. coli*. The peripheral blood count at this time showed pancytopenia with myeloblasts. The last phase of the disease characterized by myeloblastosis and septicemia was reflected in the changes observed in the serum B_{12} levels and the B_{12} binding proteins. The most important change was the decrease in the TC I and increase in the TC II associated with reduction in the bound serum B_{12}. The latter findings were observed in hepatocellular damage [14].

Comment

The observations [illegible] the significance of the serum B_{12} concentration and TC I [illegible] in the absence of any other diagnos-



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Fig. 1. The epidural tumor macroscopically

Fig. 2. Histology of the tumor. HE × 400



Fig. 3. Imprint of the tumor showing 3 blasts. May-Grunwald-Giemsa. × 1,000.

logical signs occurred with the development of spastic paraparesis and urinary incontinence. On myelography complete obstruction was found at the level of D9. Laminectomy was performed and an epidural vascular, greyish-yellow tumor was removed. Sections and imprints revealed a tumor consisting of mature eosinophils, eosinophilic myelocytes and blast cells, a picture compatible with myeloblastoma (fig. 3). No neurological improvement was obtained after removal of the tumor and the spinal column was irradiated with a total dose of 2,400 rad. This therapy did not improve the neurological signs. Pancytopenia was found subsequently. Bone marrow aspiration at this stage showed hypercellular marrow with myeloid-erythroid ratio of 20:1, due to granulocytic hyperplasia. Almost all the cells were blasts and promyelocytes. No Auer bodies were found.

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Comments

The observations demonstrate the significance of the serum B_{12} concentration and TC I determination in the absence of any other diagnos-

tic criteria of myeloid leukemia. The possibility that marked eosinophilia associated with different conditions other than leukemia was excluded when sera of patients with eosinophilia of different causes (parasites, allergy, etc.) were found to have no abnormal vitamin B_{12} parameters. The latter findings were corroborated also in reported cases of marked eosinophilia associated with tumor [17]. Leukemic eosinophilia with high serum B_{12} values were reported in a few cases [6].

Our findings clearly demonstrate that the eosinophilic granulocytes have the capacity to produce excessive amounts of TC I, similar to neutrophilic granulocytes [12]. The lack of parallelism between the peripheral white cell count and the levels of TC I and B_{12}, as observed in other cases of CML, is demonstrated in this case as well. It is therefore apparent that the TC I producing cells are present in the organs where the myeloid cells proliferate, namely the bone marrow and the spleen. The determination of serum B_{12} binders is important in the differentiation between high serum B_{12} due to hepatic injury and to myeloid proliferation.

Myeloblastoma [18] as a complication of eosinophilic leukemia was described and 6 cases of myeloblastoma occurred in a series of 65 cases of eosinophilic leukemia [10]. In some of these cases the diagnosis of leukemia was made only on the basis of the complication of myeloblastoma. Some of these cases are reported as chronic eosinophilia preceding myeloblastoma [11].

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S. SUMIDA: **Transfusion of Blood Preserved by Freezing.** Thieme, Stuttgart 1974. 92 pp., 117 fig., 15 cpl., 11 tab. DM 58.–

Der mit vielen, zum Teil farbigen Fotografien und Zeichnungen versehene Band gibt eine gute Übersicht über den heutigen Stand der Kenntnisse über die Herstellung, Lagerung und Transfusion von gefrorenen Erythrozyten und Thrombozyten. Ein langer Abschnitt ist dem eigentlichen Gefrier- und Auftauvorgang von Erythrozytensuspensionen in physiologischer Kochsalzlösung und in Glyzerin gewidmet. Die Technik der Blutkonservierung ist sehr ausführlich mit detaillierten Vorschriften behandelt und mit vielen, zum Teil allerdings wenig instruktiven und unnötigen Bildern erläutert. Die Aufzählung und Beschreibung der nötigen Analysen der Blutkonserven, insbesondere der Transfusionstauglichkeit der Erythrozyten nach der Auftauung sowie der prätransfusionellen Serologie zeigt, dass hier ein wesentlich grösserer Aufwand getrieben werden muss als bei den nach herkömmlicher Art gelagerten Transfusionseinheiten. Ein Kapitel enthält die Erörterung der Vorteile bei Verwendung von gefrorenem Blut gegenüber der Verwendung von konventionell gelagertem Blut. Der grösste Teil dieser Vorteile lässt sich allerdings in einem gut organisierten Spendezentrum auch ohne die sehr teure Zellgefrierung erreichen. Es ist deshalb wohl etwas übertrieben, wenn auf dem Klappentext des Buches vermerkt wird: «It is therefore only a matter of time before frozen blood will become an integral part of every bloodbank». Das Schlusskapitel befasst sich mit dem Gefrieren von Blutplättchen, insbesondere auch mit Funktionsprüfungen eingefrorener und wieder aufgetauter Thrombozyten, einschliesslich der klinischen Wirksamkeit solcher Präparate. Der Band kann allen empfohlen werden, die sich mit der Kryobiologie der Blutzellen befassen, speziell Medizinern und Biochemikern, die sich in Blutspendezentren mit Problemen der Herstellung, Lagerung und Administration von gefrorenen Zellen konfrontiert sehen. R. PFLUGSHAUPT, *Bern*

S. SHERRY and A. SCRIABINE (eds.): **Platelets and Thrombosis.** Urban & Schwarzenberg, München 1974. 332 pp., 120 fig.

This book represents the printed version of a symposium held in Philadelphia. The problem of thrombosis and the role of platelets are becoming more and more important. Many of the twenty articles are completed by an abundant list of references. In this field investigations are progressing rapidly and the various aspects have been taken into consideration.

An interesting attempt has been made by P. N. WALSH to integrate the blood coagulation and haemostatic processes. In special contributions the storage in platelets and the release reactions have been carefully reviewed. The inhibition of platelet function, particularly of their aggregation, has been examined and the role of the cyclic AMP and prostaglandins is discussed. It still remains to be established if the experimental data are relevant as regards the *in vivo* role of platelets. Anyhow the accumulated data will greatly influence the future progress of our knowledge concerning the platelet physiology. Other chapters are more directly

concerned with practical problems such as inhibition of platelet aggregation after renal transplantation or with subjects not primarily related to platelets such as defibrination with Arvin.

Platelets and thrombosis, edited by S. Sherry and A. Scriabine, presents an up-to-date view of the recent development in this field and a very valuable source of information. F. Duckert, *Basel*

Elmer B. Brown (ed.): **Progress in Hematology**, vol. VIII. Grune & Stratton, New York 1973. 322 pp. US $ 22.50.

This volume of the series contains chapters on ion and water movements in red blood cells (E. P. Orringer and J. C. Parker), clinical usefulness of specific antiglobulin reagents in autoimmune hemolytic anemias (H. Chaplin, jr.), acquired disorders of hemoglobin (T. B. Bradley and H. M. Ranney), dyserythropoiesis and dyserythropoietic anemias (S. M. Lewis and R. L. Verwilghen), hereditary disorders of the red cell in animals (S. M. Bannerman, A. Edwards, P. H. Pinkerton), immunologic properties on antihemophilic factor (L. W. Hoyer), regulation of myelopoiesis as approached within *in vivo* and *in vitro* techniques (F. Stohlman jr., P. J. Quesenberry, W. S. Tyler) and human marrow transplantation, current status (C. D. Buckner, R. A. Clift, E. D. Thomas, *et al.*), correlations of *in vivo* and *in vitro* measurements of hemolysis in hemolytic anemia due to immune reactions (W. F. Rosse). G. Rosenow, New York, N.Y.

Competition

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